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Fast Determination of Human Insulin and Its Deamidation Product by Electrospray Ionization Mass Spectrometry

Teerapat Rojsajjakul
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Teerapat Rojsajjakul entitled "Fast Determination of Human Insulin and Its Deamidation Product by Electrospray Ionization Mass Spectrometry." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Chemistry.

Kelsey D. Cook, Major Professor

We have read this thesis and recommend its acceptance:

Michael Sepaniak, Charles Feigerle

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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FAST DETERMINATION OF HUMAN INSULIN AND ITS DEAMIDATION PRODUCT BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Teerapat Rojsajjakul

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ABSTRACT

Application of the electrospray ionization (ESI) technique in mass spectrometry (MS) has played an important role in analysis of biological molecules and polymers. The focus of this thesis is to assess and extend the application of this technique in the rapid determination of human insulin and its deamidation product.

Quantitative assessment of deamidation of human insulin generally invokes relatively slow chromatographic analysis. Faster cost-effective analysis using mass spectrometry with a low-resolution analyzer is challenging because the deamidation product is only one Dalton heavier than the 5808-Da protein; low-level contamination has only a small effect on the isotope envelope. Exploiting the chemical differences between insulin and its deamidation product (6 carboxylic acids, vs 7 following deamidation), we have extended an application of an alternative MS method based on the relative intensities of the high charge states in negative ion electrospray, using isopropylamine (IPA) to enhance anion intensities. Source parameters were optimized for high sensitivity and stability of higher charge states.

The intensities of the higher charge states were increased in proportion to the amount of the deamidation product present in the mixtures. From the various ratios of the deamidation product, the study shows that this method was able to determine the ratio of the deamidation product in one minute with LOD = 0.7 % deamidation.

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Application of the electrospray ionization (ESI) technique in mass spectrometry (MS) has played an important role in analysis of biological molecules and polymers. The focus of this thesis is to assess and extend the application of this technique in the rapid determination of human insulin and its deamidation product.

Quantitative assessment of deamidation of human insulin generally invokes relatively slow chromatographic analysis. Faster cost-effective analysis using mass spectrometry with a low-resolution analyzer is challenging because the deamidation product is only one Dalton heavier than the 5808-Da protein; low-level contamination has only a small effect on the isotope envelope. Exploiting the chemical differences between insulin and its deamidation product (6 carboxylic acids, vs 7 following deamidation), we have extented an application of an alternative MS method based on the relative intensities of the high charge states in negative ion electrospray, using isopropylamine (IPA) to enhance anion intensities and source parameters were optimized for high sensitivity and stability of higher charge states.

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ABBREVIATION

Amino Acid	Three-Letter Abbreviation	One-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

CHAPTER 1

INTRODUCTION: HUMAN INSULIN AND ITS SEPARATIONS

Insulin is a pancreatic hormone protein secreted by the beta cells of the islets of Langerhans. It is essential for the metabolism of carbohydrates and the regulation of glucose levels in the blood, an inadequate level of insulin in the blood results in the onset of diabetes mellitus [1]. Human insulin is composed of 51 amino acid residues and has a molecular weight of 5808 Da. It consists of two polypeptide chains, A and B, linked together by disulfide bonds. Chain A has 21 amino acid residues and an intrachain disulfide, which links between position A6 and A11. The chain B polypeptide has 30 amino acid residues. Two interchain disulfide bonds are connected between A7 and B7, and between A20 and B19. The sequence of the insulin is shown in Figure 1.1 [1].

The comercial production of human insulin is a relatively large scale process because the need for medical insulin is several tons a year [2]. Purity of insulin is very important both in quality control in pharmaceutical manufacturing and for patients. The

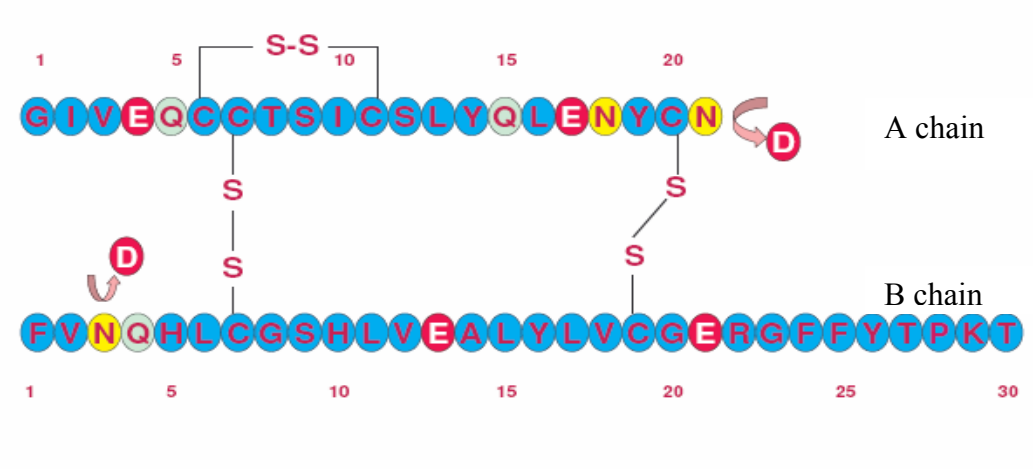

Figure 1.1 The sequence of human insulin and the deamidation sites.

purer the insulin the better it is for clinical efficacy and safety of patients. It is generally known that impurities in insulin are responsible for the immunogenicity of recrystallized insulin in patients. Most patients who receive insulin for long periods develop antibodies to insulin. In fact, these antibodies are more related to the impurities of insulin than the active sites of insulin.

The most common side reaction in manufacturing human insulin is deamidation, which may occur in both acidic and neutral solution [3]. This is because asparagine (Asn) and glutamine (Gln) are acid labile, meaning they can be hydrolyzed in acidic solution and become aspartic acid (Asp) and glutamic acid (Glu), respectively. The reaction can occur faster at high temperature. In human insulin, there are three asparagine residues at A18, A21 and B3, and three glutamine residues at A5, A15, and B4. Most complication arises when amino acid A21 is hydrolyzed and the amide group is changed to carboxylic group. As a consequence, the deamidation product of human insulin may affect the receptor binding sites and immunogenicity in the human body. Even though insulin prepared by biotechnological or chemical techniques is less contaminated than in the past, the deamidation product is an unwanted side product and difficult to separate from the target materials. The deamidation reaction is shown in Figure 1.2.

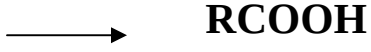

Figure 1.2 Deamidation reaction

During storage of human insulin in acidic solution, the deamidation product from A21 of human insulin is a major product while the deamidation and racemization reaction at B3 may dominate in neutral solution [4]. In addition to the deamidation reaction, human insulin may undergo covalent cross-linkage forming dimers and higher molecular weight transformation products, which decrease the activity of the native insulin.

1.1. Separation of human insulin and its deamidation product

Chromatography is the common method for separation and detection of the deamidation product. For many years, in this application, reversed phase high performance chromatography (RP-HPLC) has been a standard tool in separations because of its versatility and wide dynamic range. For example, Eli Lilly uses a C8 RP-HPLC for routine determination of insulin and its deamidation product. However, this method is relatively slow, making it less than ideal for the needs of pharmaceutical manufacturing.

1.2 Reversed Phase High Performance Chromatography (RP-HPLC)

Liquid chromatography has become one of the most important tools in separations. It is suitable for most analytes that are not sufficiently volatile for gas chromatography. HPLC in particular has been developed and has become a standard tool for both quantitative and qualitative analysis. In this system, high pressure is applied to deliver solvent (mobile phase) through a close-packed column of fine particles or chemically cross-linked substance (stationary phase). The strength of the mobile phase, type of the stationary phase, and pressure play important roles in the quality of the separations. There are many types of stationary phases for HPLC, which have different

separation mechanisms such as ion-exchange, molecular exclusion, affinity and adsorption chromatography (including reversed phase chromatography).

RP HPLC is a separation technique that uses the hydrophobic interactions between the analyte and the stationary phase. In general, non-polar alkyl chains are covalently attached to a silica surface to make fine particles for the packed column. The most common non-polar stationary phases are shown below.

$R = (CH_2)_3CH_3$ Butyl group (C4)

$R = (CH_2)_7CH_3$ Octyl group (C8)

$R = (CH_2)_{17}CH_3$ Octadecyl group (C18)

$R = C_6H_5$ Phenyl group (where R is an alkyl chain).

This interaction mechanism can provide high resolution for separation. In fact, RP-HPLC is able to separate proteins of nearly identical sequences, such as insulin variants, which differ by a single amino acid. For proteins, the separation relies on the interaction between the amino acid side chains and the stationary phase. Under typical optimized conditions, the separation time for human insulin and its deamidation product by C8 RP-HPLC is about 20 minutes, which is relatively slow.

Improvement of RP-HPLC, has been reported by using an isotherm approach [4]. In this study, human insulin and its deamidation product were separated by pulsed mobile phase experiment using ODS-AM C18 stationary phase with a particle diameter of 5 microns. An ethanol-water mixture (29.8%: 70.2% (w/w)) was used as the mobile phase,

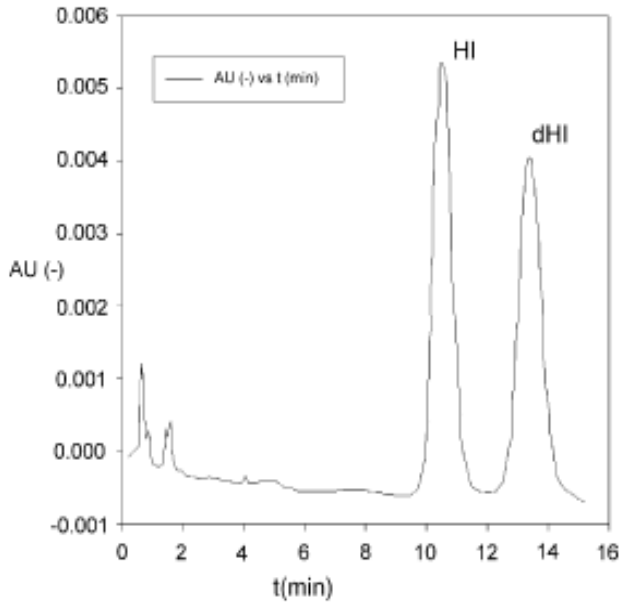

Figure 1.3 Separation of human insulin and its deamidation product in a pulsed experiment with C18 RP-HPLC [4].

buffered with 50 mM ammonium sulfate at pH 3.5. In this case, human insulin and its deamidation product were eluted at 10.2 and 13.0 min, respectively. The chromatogram of this separation is shown in Figure 1.3.

1.3 Reversed Phase Ultra High Performance Chromatography (RP-UPLC)

With the same mechanism as RP-HPLC, reversed phase ultra-high performance chromatography (RP-UPLC) operates under ultra-high pressure to perform the chromatographic separation [5, 6]. This system has advantages for speed and capability of operation at a maximum pressure up to 15000 psi (1000 bar). Separations at ultra-high pressure can obtain efficiencies of greater than 200000 theoretical plates/meter. However, RP-UPLC is relatively new. Thus the stationary phases for this type of

separation are in development. Many factors need to be considered for short column packing with small particles such as:

Small volume device (for low band spreading)

Fast, efficient detector (for example, a mass spectrometer and a fluorometer)

Advanced stationary phase and packing techniques

Hardware and software

For separation of human insulin and its deamidation product, RP-UPLC is a new candidate for an alternative method when speed is required. However, reproducibility is another issue that needs to be improved. The separation time for human insulin and its deamidation product by RP-UPLC is in a process of development.

CHAPTER 2

ELECTROSPRAY MASS SPECTROMETRY AND GAS PHASE CHEMISTRY

2.1. Electrospray Ionization Mass Spectrometry

Electrospray ionization (ESI) is a method by which the analytes present in solution can be ionized and transferred to mass analyzers. This indispensable method is a "soft ionization" technique because it allows detection of intact molecular ions of large, nonvolatile species. These are generated from a solution in a small capillary by applying a potential difference of 3-6 kV between the capillary and a counter electrode under atmospheric pressure. The concentration of the analyte (generally low in a volatile solvent) can be micromolar or lower depending on types of mass analyzers, analytes and the detectors. The experiment and the characteristics of ESI were pioneered by Dole in the late 1960s [7].

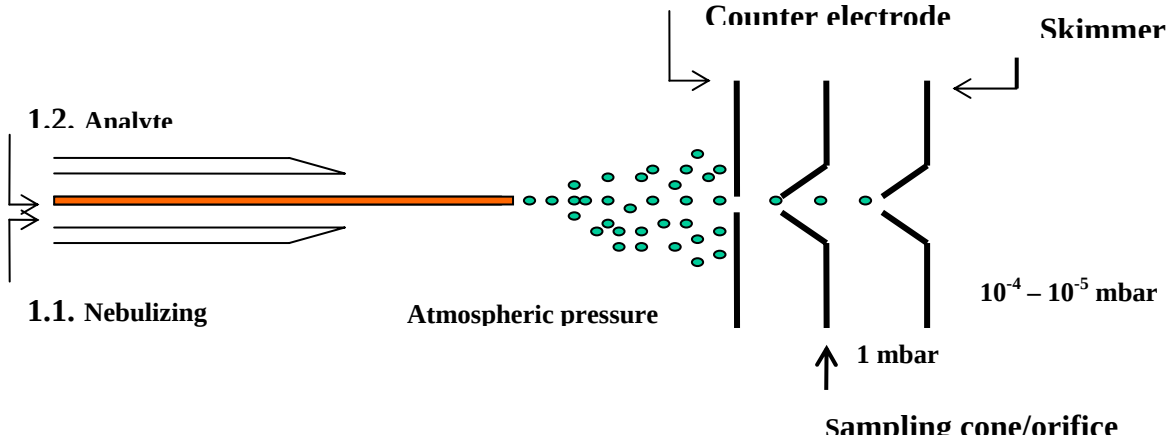

Figure 2.1 Standard electrospray ionization source

Later, electrospray ionization was developed for the analysis of macromolecules, including biolomolecules and polymers, by Fenn, who was awarded for the Nobel Prize

in Chemistry in 2002 [8].

In general, the formation of ions in ESI involves three main steps: first, the formation of charged droplets, second, the evaporation of solvent, resulting in shrinkage of the droplet and third, formation of smaller charged droplets and ions in gas phase. As the analyte (usually in a volatile polar solvent) is pumped through narrow capillary, (usually made of stainless steel), a high voltage (3-6 kV) is applied to the tip of the capillary, producing a spray of highly charged droplets. As the solvent evaporates, the droplets became smaller, finally disintegrated into many small charged droplets without the solvent. These small charge droplets move through the orifice by the difference of voltage and pressure.

The unique characteristics of ESI mass spectra allow for the production of ions

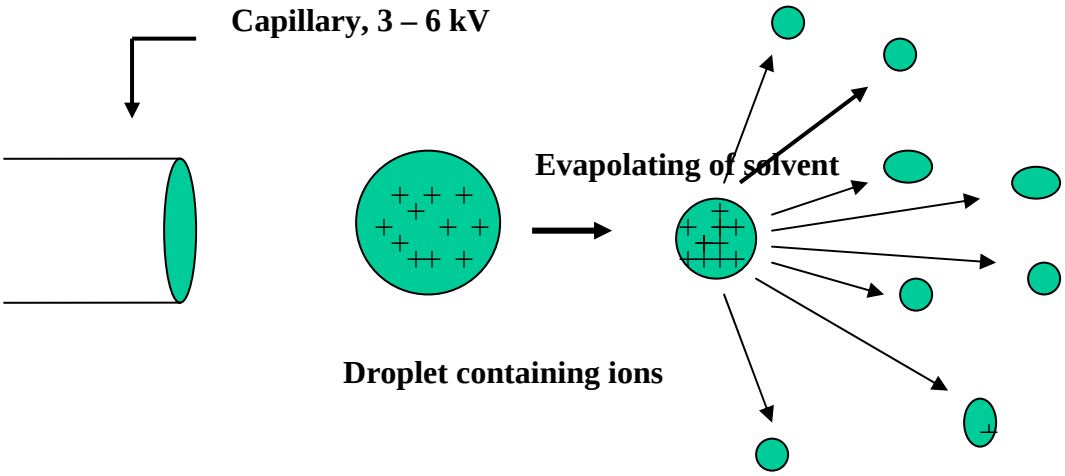

Figure 2.2 The electrospray ionization process.

with multiple charge states, which depend upon the characteristics of the structure, conformation and functional groups of the analytes.

2.2. Gas Phase Chemistry in ESI

Dole et al. [7] studied negative macroions of a dilute polymer solution, which was produced by sampling the gaseous mixture of macroions, solvent, and nitrogen molecules with a nozzle-skimmer system. The results showed that polystyrene macroions with average molecular weight of 51 000 Da tend to form dimers and trimers in the beam, and the larger polystyrene macroions, which have an average molecular weight of 411 000 Da, are most likely to form multiply charged single species. This is because during the transit of the macroions in the supersonic beam, the ions become more concentrated due to the higher volatility of solvent. However, the reason why decreasing mass-to-charge ratios of high molecular mass solutes yield higher charged ions was not clear at that time [8]. To explain this problem, John B. Fenn, suggested that the charge state distributions of noncovalent macromolecule clusters, either of proteins or polymers, are related to their droplet charge densities [9]. These charge densities result from the rate of desorption in the ionization process, such that the number of charge states depends on the number of charges on the droplet surface. This generally explains why higher mass-to-charge ratios have fewer charge states or the other way around.

In practice, the number of charge states in biomolecular ESI depends upon the number of acidic sites in proteins or peptides in positive ion ESI [10], and the number of basic sites in negative ion ESI, and relates to protonation and deprotonation of those sites in ESI. In this regard, the protonation of amino acids lysine, arginine and histidine yield positive ions. In negative ESI, the deprotonation of aspartic acid and glutamic acid yield negative ions. In some proteins, the maximum number of charges is decreased because

of disulfide bonds [11]; in the compact conformation of proteins induced by disulfide linkages, both basic and acidic sites can be protected. This will shift the charge states to lower number in ESI.

Environmental factors also play an important role in the charge state distribution. The distribution can be manipulated depending on many factors such as pH, solvent, concentration, additives, structures, conformations and instrument conditions. The most sensitive variable affecting the charge state distribution is the pH of the solution.

A convenient method for manipulating charge state distribution was demonstrated by Leenheer [12], who developed a method for quantitative analysis for analysis of proteins. In this study, human insulin was used for the model of charge distribution under conditions of varying pH in solution and varying instrumental conditions. The study showed that gas phase formic acid, introduced via a bubble trap in the nebulizing gas line promoted the highest positive charge state of human insulin in postive ion ESI. Conversely, gas phase isopropylamine enhanced the highest charge state of human insulin in negative ion ESI. In addition, the instrumental parameters, such as cone voltage, capillary temperature and the flow rate of the nebulizing gas were optimized for the best distribution of the charge states. The results from this study are shown in Figure 2.3 [12].

The experiment carried out and reported in Figure 3D, is “wrong-way-round” ESI

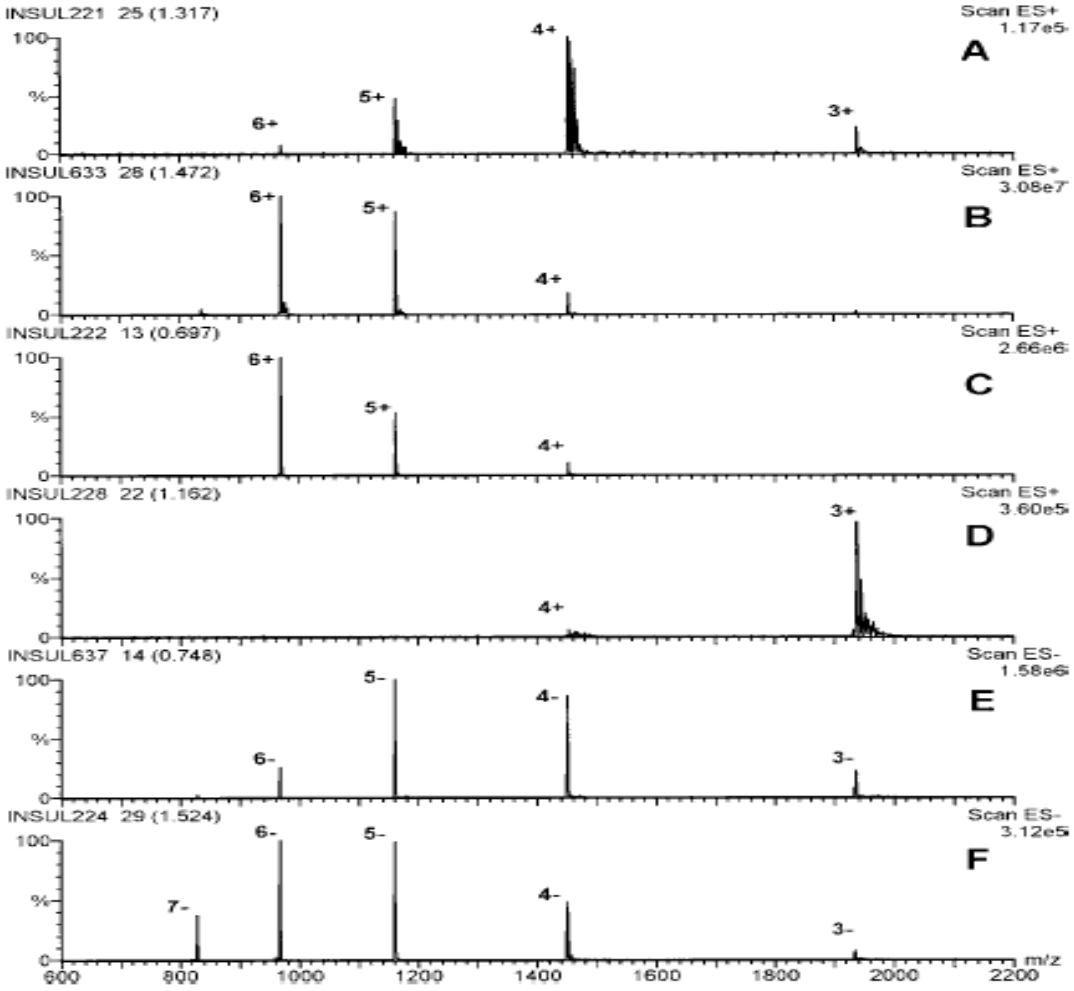

Figure 2.3 Positive and negative ion electrospray ionization mass spectra of human insulin; in positive ESI: with neutral solution (A), with 1% formic acid solution (B), with gas phase formic acid (C), with 0.003% formic acid and gas phase isopropylamine (D); in negative ion ESI: with 1% isopropylamine (E) and gas phase isopropylamine (F).

[13], in which the positive ion ESI was operated under basic conditions. Under these conditions, the lower positive charge states were expected to be favorable. Generally, in negative ion ESI, gas phase isopropylamine gives better sensitivity, compared to the solution phase and the charge states shift towards the higher charge states.

2.3. Electrical Discharge (Corona Discharge)

Corona discharge is a charge reduction process in ESI, resulting in a change in appearance of the mass spectrum [13, 14]. It usually takes place between the capillary and counter electrode. This phenomenon can reduce the charge states induce fragmentation and/or prevent observation of many ions in ESI. Corona discharge can take place very readily under a negative potential at the spray tip of capillary. This is because the field emission of electrons from the tip does not require a very high voltage. In this process, both positive and negative charge carriers are formed and recombine with droplets having the opposite charge [15]. As a result, droplet charges are neutralized and the ions for most analytes no longer appear in mass spectra. Even though these ions are sensitive to corona discharge and usually disappear in mass spectra, this problem can minimized by adjusting parameters of the mass spectrometer such as lowering the electric field.

A low-resolution mass spectrometer does not provide enough resolution to distinguish human insulin and its deamidation product by regular ESI. This is because deamidation induces only a 1 Da change in mass ($CONH_2 \rightarrow COOH$) out of a total mass of 5808. Thus, direct MS determination is difficult at low resolution due to interference

between the deamidation product and the ^{13}C isotopic peaks of the native protein.

Our goal is to extend the gas phase isopropylamine method [12] to determine the ratio of human insulin and its deamidation products in negative ion ESI for high speed and throughput. This is because the current method, RP HPLC, which has been used for decades in the pharmaceutical industry, is relatively slow.

CHAPTER 3

EXPERIMENTAL METHODS

3.1. Materials:

HPLC-grade acetonitrile (MeCN) and water were purchased from Fisher Scientific (Pittsburgh, PA). Reagent-grade formic acid (95% in water) and trifluoroacetic acid (TFA) were purchased from Pierce Biotechnology Inc. (Rockford, IL). Formic acid (FA) and human insulin were purchased from Sigma Chemicals (St. Louis, MO). Isopropylamine was purchased from Eastman Organic Chemical (Rochester, NY). All reagents were used without further purification.

3.2. Preparation of the Deamidation Samples:

There are two sets of samples were used in this study. All were prepared from stock solutions of human insulin (3 mg/mL in 0.1 aqueous formic acid). In Set 1, a stock solution was aged for 2 months at room temperature to promote deamidation. Aliquots of this solution were mixed with aliquots a fresh solution to prepare 3 samples (1 mL) of varying deamidation concentrations, labeled sample 1, 2 and 3. In Set 2, a stock solution of human insulin was aged for 4 months at 4 °C to promote deamidation. Aliquots of this solution were mixed with aliquots a fresh solution to prepare 5 samples of varying deamidation concentrations, labeled sample 4, 5, 6, 7 and 8.

3.3. Determination of Human Insulin and Its Deamidation Product by HPLC:

The various mixtures of human insulin and its deamidation product from set 1 and set 2

were analyzed by reversed phase HPLC using a Hewlett-Packard (Palo Alto, CA) 1100 system with a C8 reversed phase column (4.6 mm x 150) at 25 °C. Solvent A was 0.05% TFA in water, and solvent B was 0.05% TFA in MeCN. The flow rate through the column was 1.0 mL/min. Each 20 μL (approximately 10 nanoMolar) sample was injected, using a Rheodyne 8125 injector (Alltech, Deerfield, IL). The column was equilibrated at 10% solvent B prior to injection. For set 1, the sample was eluted with a linear gradient from 10 to 30% solvent B over a 45 min-period; for Set 2, the linear gradient was from 10 to 20% solvent B over 45 min. The longer gradient in Set 2 was for a better separation. The separation was monitored by absorbance 280 nm. ChemStation software (Hewlett Packard, Palo Alto, CA) was used to determine peak areas.

3.4. Determination of Human Insulin and Its Deamidation Product by Mass Spectrometry:

A Micromass (Manchester, UK) Quattro II triple quadrupole mass spectrometer was used to acquire ESI mass spectra. An HPLC in-line bubble trap (Alltech, IL, US; part no. 01-0221, 1/16") was inserted in the nebulizing gas line in a "head-down' manner [12].

The chamber of the bubble trap is screw-capped with a 200 μL plastic vial filled with isopropylamine inside (Figure 3.1). Source parameters were optimized for high sensitivity and stability for the highest charge states in the negative ion mode. The source temperature was 120 °C. Flow rates of nitrogen nebulizing and drying gas were 20 and 200 L/h, respectively. The capillary voltage was 2.50 kV, and the cone voltage was 30 V.

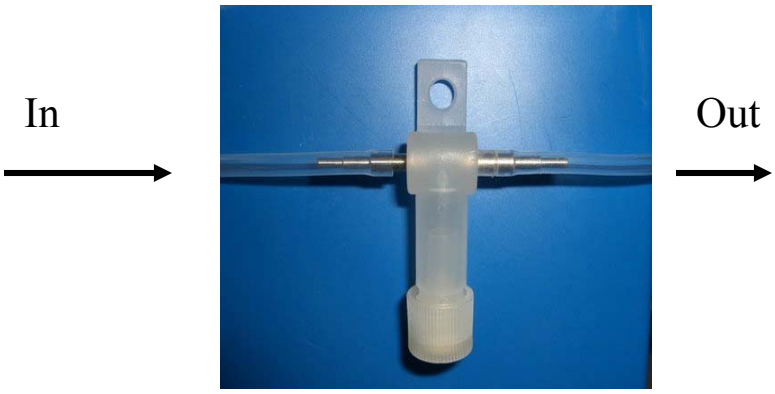

Figure 3.1 The bubble trap in the standard nebulizing gas line of the Quattro II mass spectrometer.

About 2 μL of each mixture were diluted with 1mL 50% ACN: H_2O. Each sample was infused to the ESI source by a Harvard Apparatus (South Natick, MA) syringe pump at a flow rate of 5 μL/min. Mass spectra were acquired in the accumulation mode from 700 to 1300 m/z for 1 min (17 scans) for Set 1 and 700 to 1500 m/z for 1 min (17 scans) for Set 2. Ten replicates for set 1 and five replicate for set 2 were used for each sample. The change in the range of the scan in Set 2 was to obtain more number of charge states.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Results from C8 reversed phase HPLC

The three mixtures from Set 1 were determined by C8 reversed phase HPLC to be approximately 1%, 30% and 60% hydrolyzed, respectively. The results suggest that the deamidation may simply occur either in the acidic condition or in solution at above 37°C [3]. We were interested in even smaller ratios of the demidation product in the range of the problematic for pharmaceutical manufacturing. Therefore, experiment set 2 was carried out at 4°C for 4 months. The results from C8 reversed phase HPLC for five samples were 0.9%, 5.4%, 8.6%, 11.2% and 17.1% deamidation, respectively.

It is interesting to note that the elution time of human insulin and its deamidation product does not follow the expected order based on the theory of C8 RP-HPLC. In general, the separation mechanism of reversed phase HPLC depends on hydrophobic interactions between the analyte and stationary phase. The amide group of human insulin should be more hydrophobic than the carboxyl group in the deamidation product. Therefore, human insulin should have a stronger interaction and elute after the deamidation product. On the contrary, in this separation, the deamidation product elutes after the human insulin. The results suggest that deamidation changes the conformation of human insulin and makes it more hydrophobic, resulting in longer retention by the C8 reversed phase, which agrees with reference [4]. An example of C8 RP-HPLC is shown in Figure 4.1.

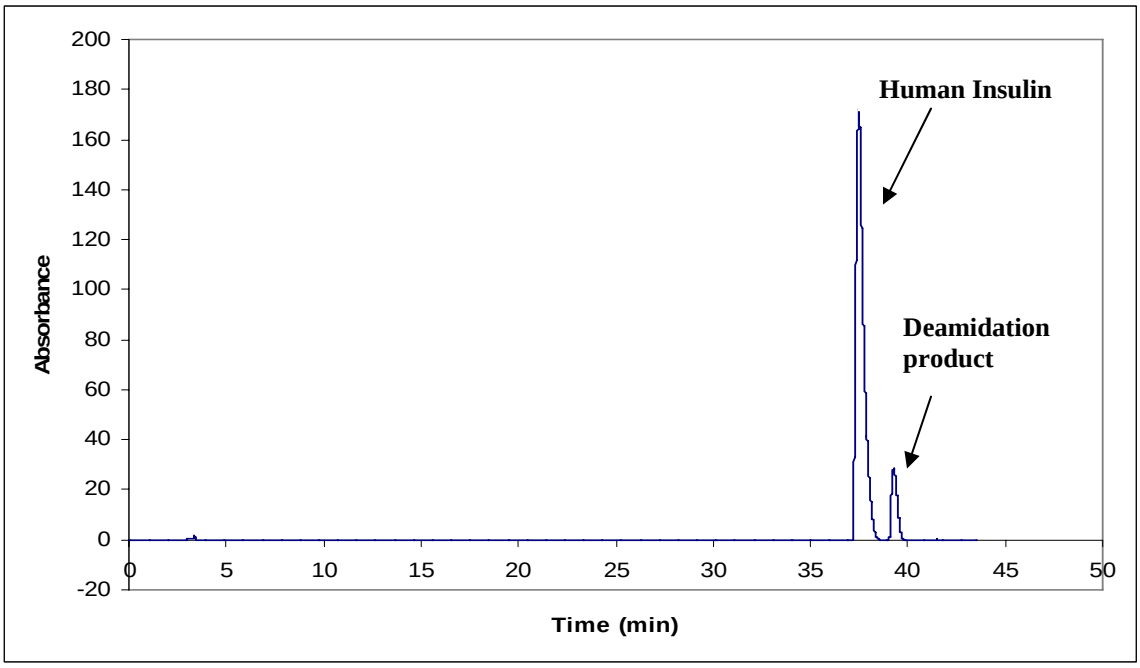

Figure 4.1 Chromatogram of sample 1 of human insulin and its deamidation product by C8 RP-HPLC, which has 11.2 % of the deamidation product.

The samples from both set 1 and 2 were analyzed by the mass spectrometric method to improve the determination speed of the deamidation product in human insulin samples.

4.2. Results from Gas Phase Isopropylamine in Negative ESI Mass Spectrometry.

Adjusting the Quattro II triple quadrupole mass spectrometer's parameters. The instrument's parameters were adjusted for high sensitivity and stability of the higher charge states. The source temperature was 120 °C. Adjusting the source temperature higher and lower than 120 °C affected the intensity of the highest charge states, which could be –7 and –8. The most sensitive parameters were the cone voltage and the flow

rates of nebulizing and drying gas. Increasing the cone voltage from 25 V shifted the spectra of human insulin to lower charge states [12]. On the other hand, lowering the voltage from 25 V increased the intensity of the higher charge states but it also affected the stability of the higher charge states. The flow rates of the nebulizing and drying gas were also important for the stability of the higher charge states. The flow rates of nebulizing and drying gas in this experiment were chosen at 20 and 200 L/h, respectively. The flow rates of the nebulizing gas and the drying gas should be optimized because isopropylamine was introduced in the nebulizing gas line. If the flow rate of the drying gas was high, the stability and the intensity of the charge states were decreased due to the decrease of the concentration of isopropylamine in the gas phase and the flow fluctuation.

ESI mass spectra of human insulin. A feature of ESI-MS of human insulin is the multiply charged ions. The possible charge states in a negative ion ESI mass spectrum depend upon the number of carboxylic acid groups in the molecule. Since, human insulin has six carboxylic acid groups, the maximum charge state should be – 6. The mass/charge ratio (m/z) of each charge state can be calculated by equation below:

$$m/z = (MW - nH)/n$$

Where MW, n and H are the molecular mass, the number of the charge state, and hydrogen on the carboxylic acid group, respectively. All possible charge states of human insulin are shown in Table 4.1.

The Quattro II triple quadrupole mass spectrometer is a low-resolution mass spectrometer, which has resolution capability of about 1,000. It is not able to differentiate

Table 4.1 All possible charge states of human insulin and * of the deamidation product in negative ion ESI.

Charge State	Calculation	Mass to charge ratio (m/z)
-1	m/z = (5808 –1)/1	5807
-2	m/z = (5808 –2)/2	2903
-3	m/z = (5808 –3)/3	1935
-4	m/z = (5808 –4)/4	1451
-5	m/z = (5808 –5)/5	1160.6
-6	m/z = (5808 –6)/6	968
-7*	m/z = (5809 –7)/7*	828.8*
-8*	m/z = (5810 –8)/8*	725.2*

isotopic clusters of higher charge states of high molecular mass proteins. In human insulin, for example, the close-ups of the –6 charge state of the samples of Set 2 are shown in Figure 4.2.

Mass spectra of human insulin with and without isopropylamine gas. Under the same parameter of mass spectrometric conditions, a negative ion ESI mass spectrum of 0.9% deamidation product in human insulin (~4 μM in 50% acetonitrile:water), without isopropylamine gas is shown in Figure 4.3.

-6

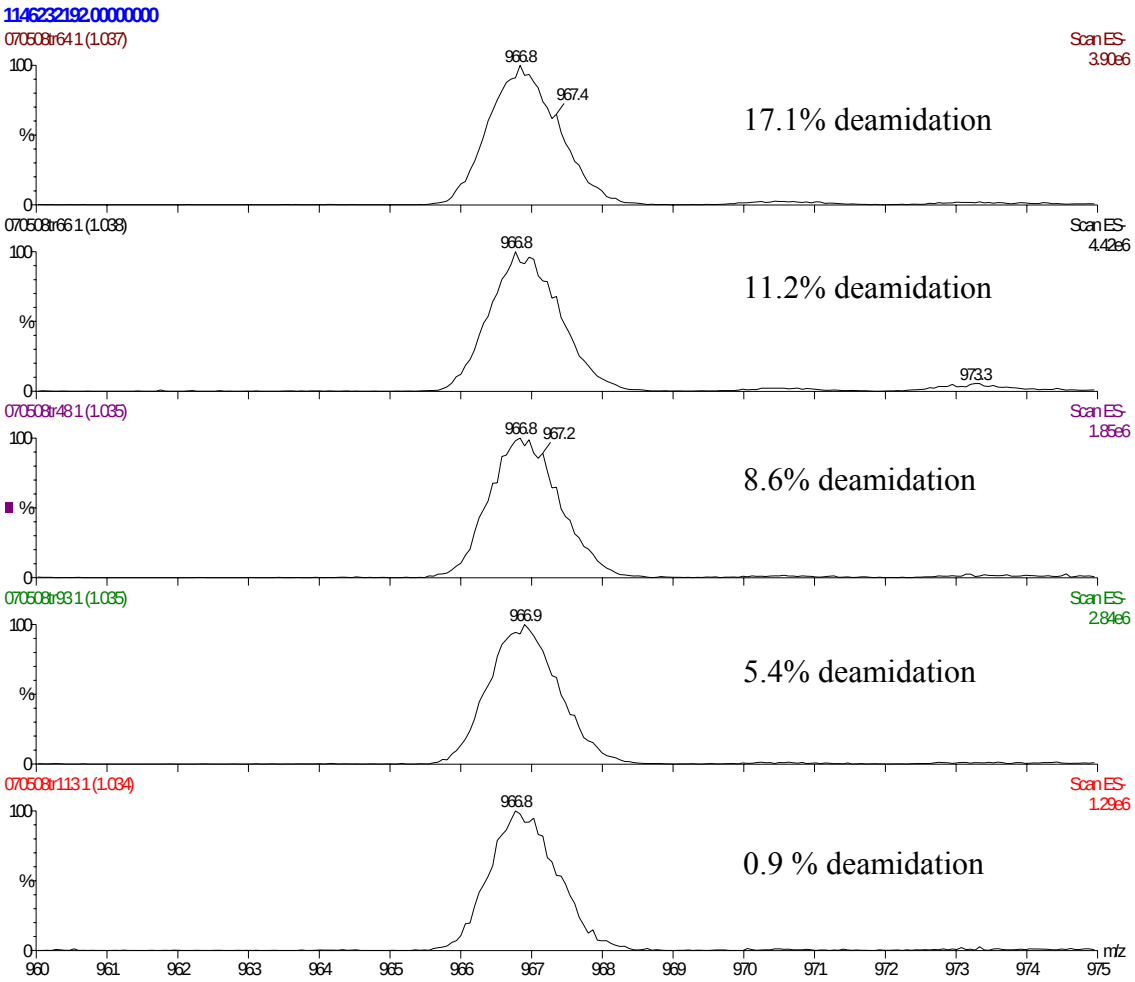

Figure 4.2 Close-up of –6 charge state of the deamidation product in Set 2

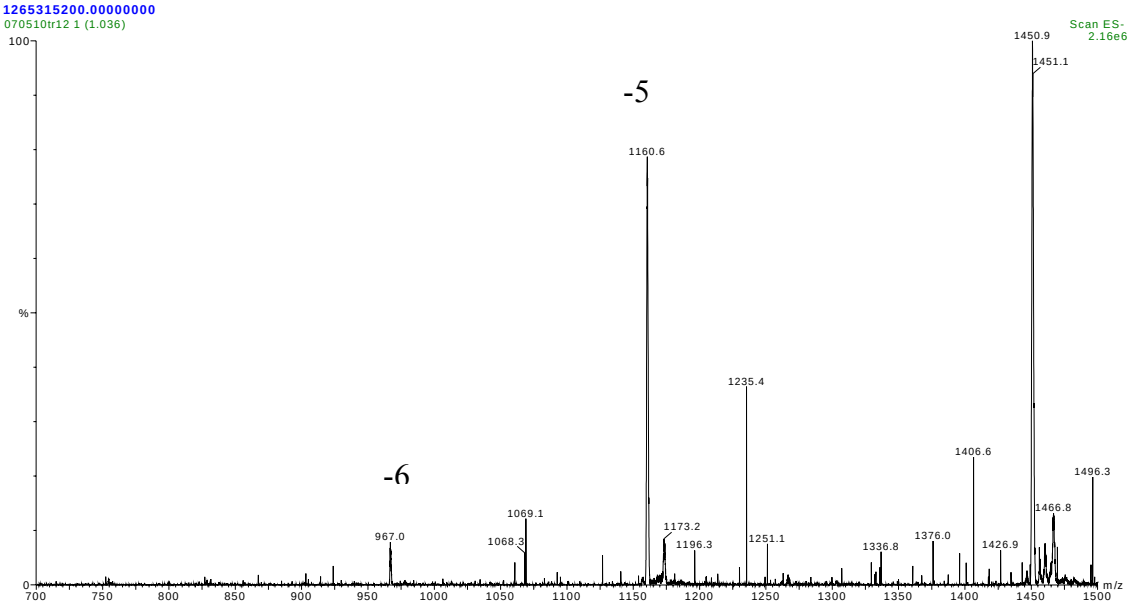

Figure 4.3 Negative ion ESI mass spectrum of 0.9% deamidation of human insulin (~4 □M in 50% acetonitrile:water), without gas phase isopropylamine

Without isopropylamine gas, the maximum charge state in negative ion ESI of 0.9% deamidation product in human insulin is –6 (m/z = 966.8). However, the relative intensity of this charge state is only about 13%. A negative ion ESI mass spectrum of 60% deamidation product in human insulin (~4 μM in 50% acetonitrile:water), without isopropylamine gas is shown in Figure 4.4.

The results show that without gas phase isopropylamine the ESI spectra of 0.9% deamindation and 60% deamidation of human insulin are slightly different. Even though the relative intensity of the –6 charge state of 60% deamidation is about 5% higher than the 0.9% deamidation, the determination of human insulin and its deamidation product will not be successful at percent of the deamidation mixture. Moreover, the stability of

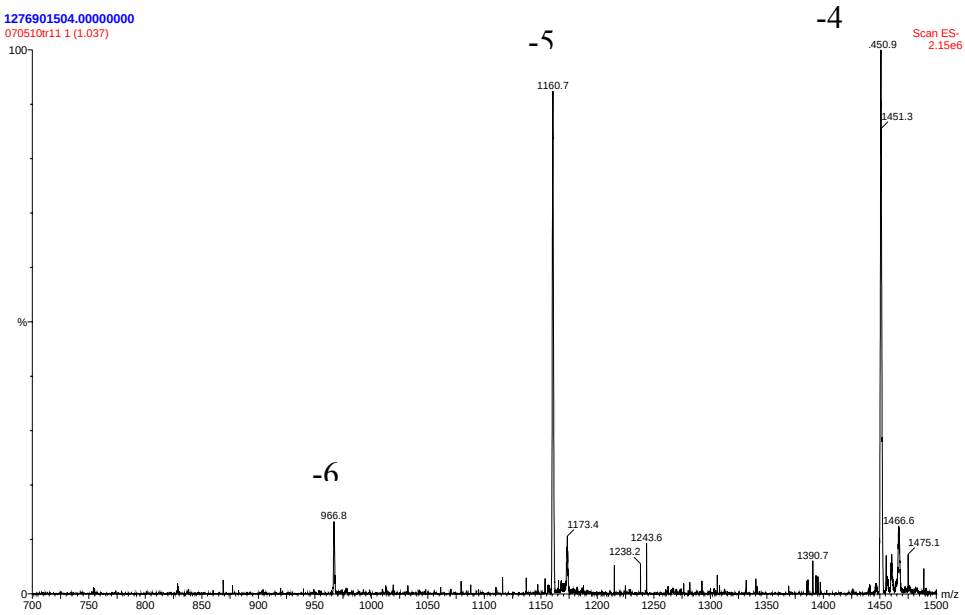

Figure 4.4 Negative ion ESI spectrum of 60% deamidation of human insulin (~4 □M in 50% acetonitrile:water), without gas phase isopropylamine

the higher charge state may also affect the measurement. The ESI mass spectrum of the 0.9% human insulin at ~ 2 μM in 50% acetonitrile:water under the same concentration and mass spectrometric conditions without isopropylamine is shown in Figure 4.5.

The negative ion ESI mass spectrum of 0.9% deamidation of human insulin in 50% acetonitrile:water shows that the signal-to-noise ratio of the –5 charge state of human insulin is less than 1. Fortunately, the negative ion ESI mass spectrum shows better signals with gas phase isopropylamine under the same mass spectrometric conditions. The ESI mass spectrum of 0.9% deamidation of human insulin at ~ 2 μM in 50% acetonitrile:water is shown in Figure 4.6.

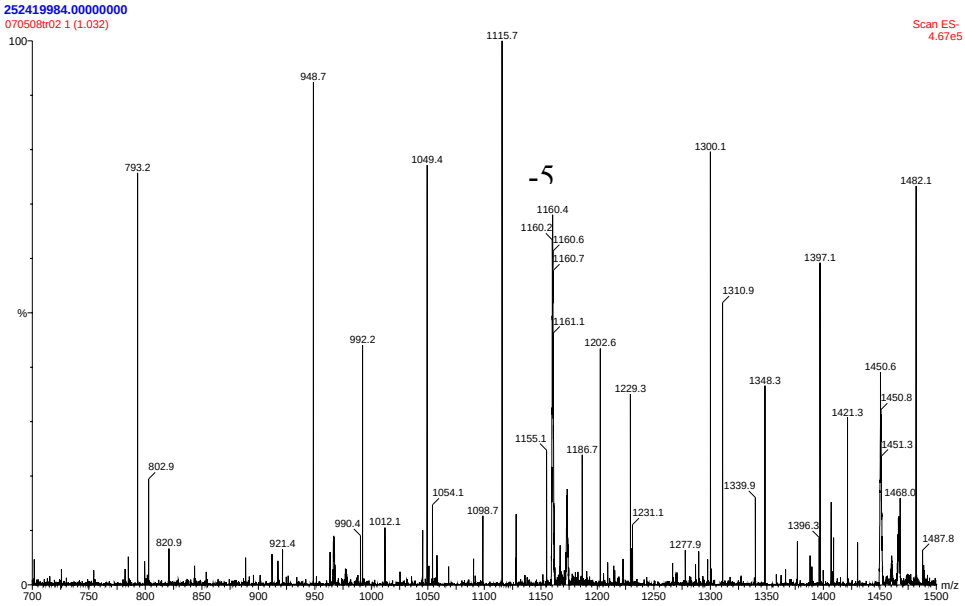

Figure 4.5 Negative ion ESI mass spectrum of 0.9% deamidation of human insulin at ~ 2 □M in 50% acetonitrile:water without gas phase isopropylamine

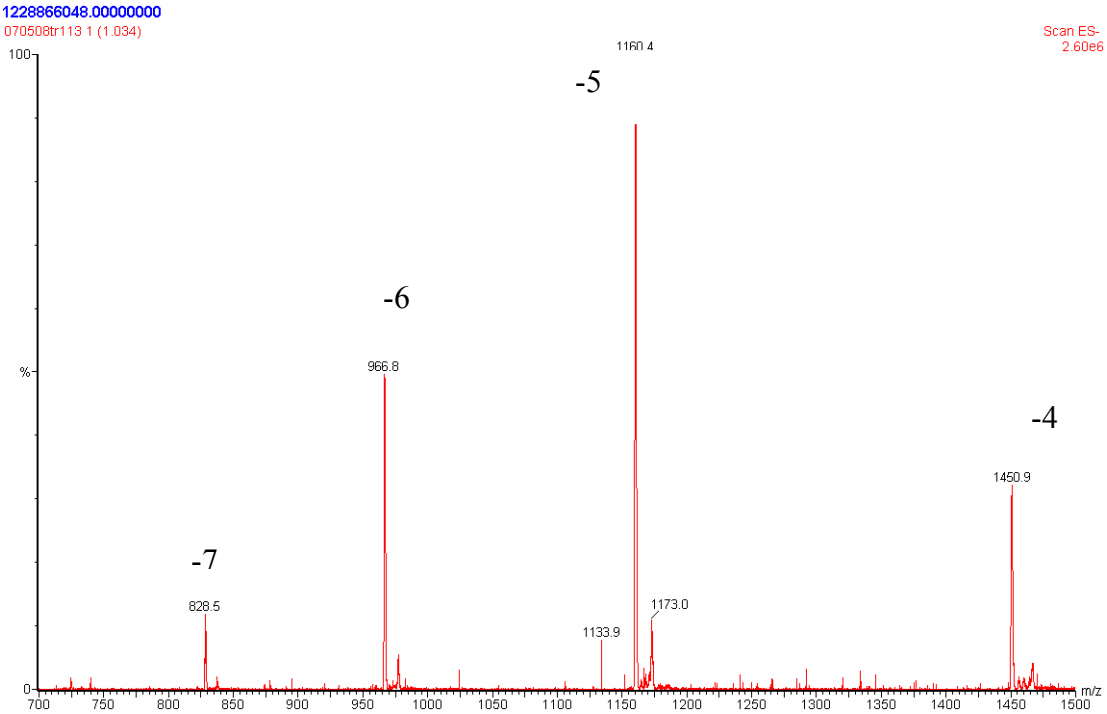

Figure 4.6 Negative ion ESI mass spectrum of 0.9% deamidation of human insulin at ~ 2 □M in 50% acetonitrile:water with gas phase isopropylamine

The concentration of insulin in Figure 4.6 is half that in Figure 4.3 but it has higher signal-to-noise ratio, suggesting that the isopropylamine gas not only promotes the higher charge states but also enhance the signals of insulin in negative ion ESI.

The study of the deamidation product Set 1. Experimental numerical data of Set 1 are shown in Appendix 1. The ESI mass spectra of the deamidation in Set 1 are shown in Figure 4.7.

The sample of Figure 4.7a contained approximately 1% of the deamidation product determined by C8 RP HPLC. The average intensity of the charge states are 0%, 12%, 72%, and 100% for charge states, -8, -7, -6, and -5, respectively. The relative intensity the charge state –7 was expected to be close to 0% because this peak should derive mainly from the deamidation product, which has more carboxylic acid groups compared to human insulin. In this case, the charge state intensity may come from either the amount of deamidation product or the response of the mass analyzer. At 30% of the deamidation product (Figure 4.7b), the average intensity for charge states –8, -7, -6 and –5 are 18%, 68%, 100%, 56% respectively. The shift of the charge states toward the higher charge states is consistent with RP-HPLC evidence indicating that the amount of the deamidation was increased during the hydrolysis in formic acid solution. This is related to the increasing the amount of carboxylic acid groups, especially at A21 and B3. In Figure 4.7c, at approximately 60% of the deamidation product, the average intensity of for charge states –8, -7, - 6 and –5 are 35%, 100%, 99% and 37%, respectively. The significant relative intensity of the charge state –8, suggests that there are at least two

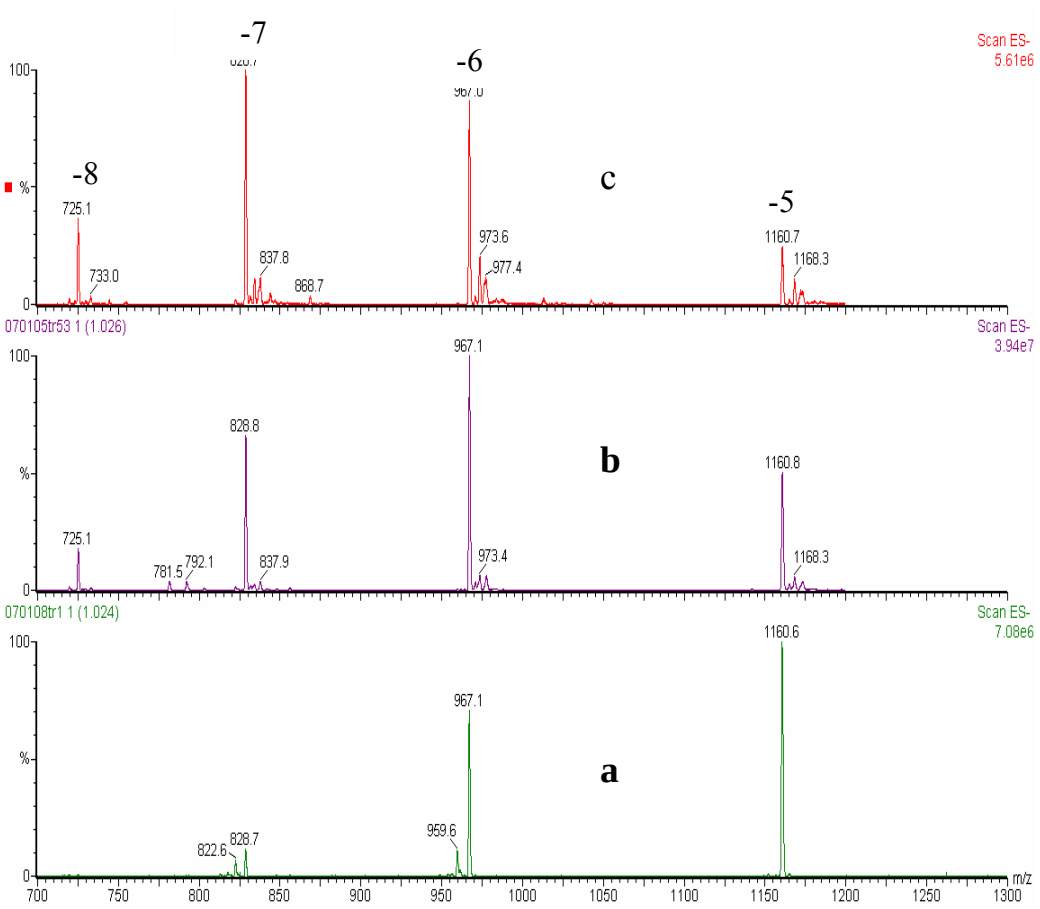

Figure 4.7 ESI Mass Spectra of human insulin samples set 1, including 1%, 30% and 60% deamidation product in 4.7a, 4.7b and 4.7c, respectively

sites of the deamidation; pure human insulin should not add to the relative intensity of the–7 or –8 charge states because it has only six carboxyl groups. To explain these results, the possible sites of the deamidation need to be considered. In human insulin, most deamidation reaction occurs at amino acid positions A21 and B3 [3]. As mentioned before, the deamidation of asparagines at A21 is favorable in acidic conditions while the reaction at B3 dominates in neutral conditions. However, both positions can be hydrolyzed to some extent under acidic or neutral conditions. Double reaction results in two additional carboxylic acid groups, can account for the –8 charge state of in the mass spectra. Moreover, other labile amino acids can also affect the intensity of those charge states. Average intensities of the charge states of human insulin and percent of the deamidation product for Set 1 are shown in Table 4.2.

The study of the deamidation product in Set 2: In set 2, samples with five different levels of deamidation (0.9%, 5.4%, 8.6%, 11.2% and 17.1% confirmed by RP-HPLC) were studied. The instrument parameters were generally the same as in Set 1 but the mass range was expanded to m/z 700 – 1500, thus the mass spectra of set 2 include the -4

Table 4.2 Average intensities of the charge states of human insulin and percent of the deamidation product for Set 1.

% Deamidation from HPLC	**% Relative intensity of charge state -8**	**% Relative intensity of charge state -7**	**% Relative intensity of charge state -6**	**% Relative intensity of charge state –5**
1.0	0 ± 0	12 ± 1	72 ± 4	100 ± 0
30.0	18 ± 2	68 ± 2	100 ± 0	56 ± 2
60.0	35 ± 2	100 ± 1	99 ± 2	37 ± 1

charge state. The relative intensity of each charge state from these two mass ranges is the same because it is only 200 Da different. However, more data points could be considered for the determination of human insulin and its deamidation product. Experimental numerical data for set 2 are shown in Appendix 2. The ESI mass spectra of the deamidation in set 2 are shown in Figure 4.8.

The average distribution of charge states intensities of human insulin and percent of the deamidation product are shown in table 4.3. Both set 1 and set 2 show that increasing the deamidation product also increases the intensity of charge state –7 and –8.

Table 4.3 Average intensity of charge states of human insulin and percent of the deamidation product for Set 2.

% Deamidation from HPLC	% Relative Intensity of charge state -8	% Relative Intensity of charge state -7	% Relative Intensity of charge state -6	% Relative Intensity of charge state -5	% Relative Intensity of charge state –4
0.9	0 ± 0	11.8 ± 6	51.8 ± 2	100 ± 0	34.5 ± 2
5.4	0 ± 0	19.2 ± 2	72.1 ± 4	100 ± 0	22.6 ± 3
8.6	4.9 ± 1	29.2 ± 3	86.2 ± 4	100 ± 0	25.0 ± 3
11.2	8.0 ± 1	38.8 ± 4	100 ± 7	84.2 ± 14	12.6 ± 2
17.1	15.6 ± 2	49.0 ± 7	100 ± 0	56.4 ± 9	6.9 ± 1

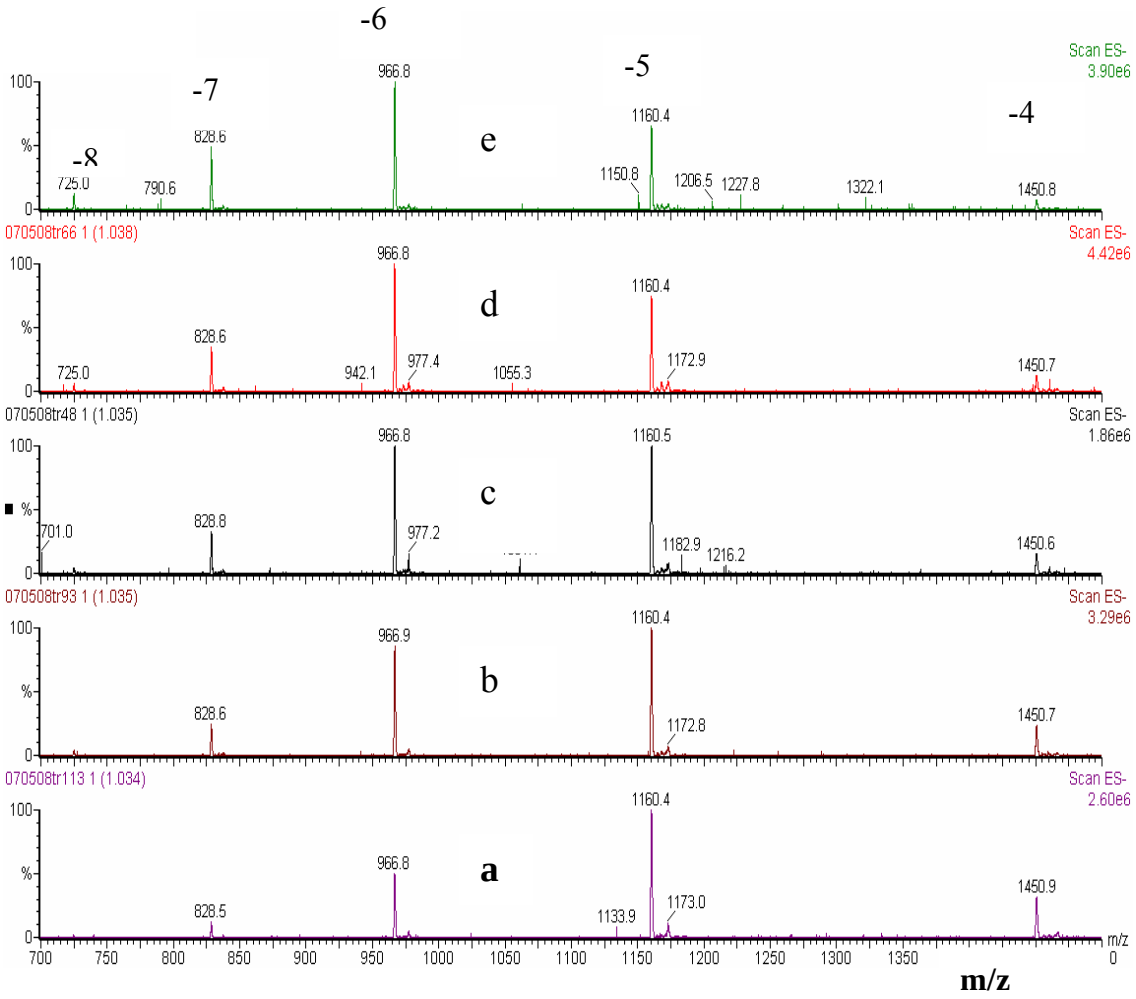

Figure 4.8 Mass spectra of human insulin samples of set 2, including 0.9%, 5.4% and 8.6, 11.2 and 17.1% deamidation product in 4.8a, 4.8b, 4.8c, 4.8d and 4.8e, respectively

4.3. Determination of the Ratio of Human Insulin and Its Deamidation Product by ESI Mass Spectrometry

The results from Set 1 and Set 2 show as the amount of deamidation product increases, the charge states distributions shift toward the higher charge states. There are many ways to construct calibration curves because there are four different charge states in data set 1 and five charge states in data set 2. Accuracy, precision, linearity and limit of detection (LOD), coefficient of variation (<CV>), and root mean square error (RMSE) depend upon which charge states are used [16, 17]. In this study, three times the standard deviation was used to calculate the ratio of the deamidation in the mixture. The quantitative analysis of data in Set 1 is shown in Appendix 1 and summarized in Table 4.4.

Table 4.4 Summary of quantitation performances of human insulin in Set 1

Numerator	Denominator	LOD	<CV>	RMSE	R^2	Plot Slope
7+8	5	0.5	0.51	26.5	0.9874	6.07
7+8	6	1.2	0.65	15.3	0.9939	2.06
7+8	5+6	0.9	0.12	5.7	0.9997	1.59
7+8	5+6+7+8	1.7	0.16	4.6	0.9674	0.73
7	5	0.7	0.17	19.7	0.9944	4.35
7	6	1.7	0.47	8.8	0.9831	1.41
7	5+6	1.2	0.42	4.9	0.9954	1.12
7	5+6+7+8	2.3	0.30	2.02	0.9343	0.50

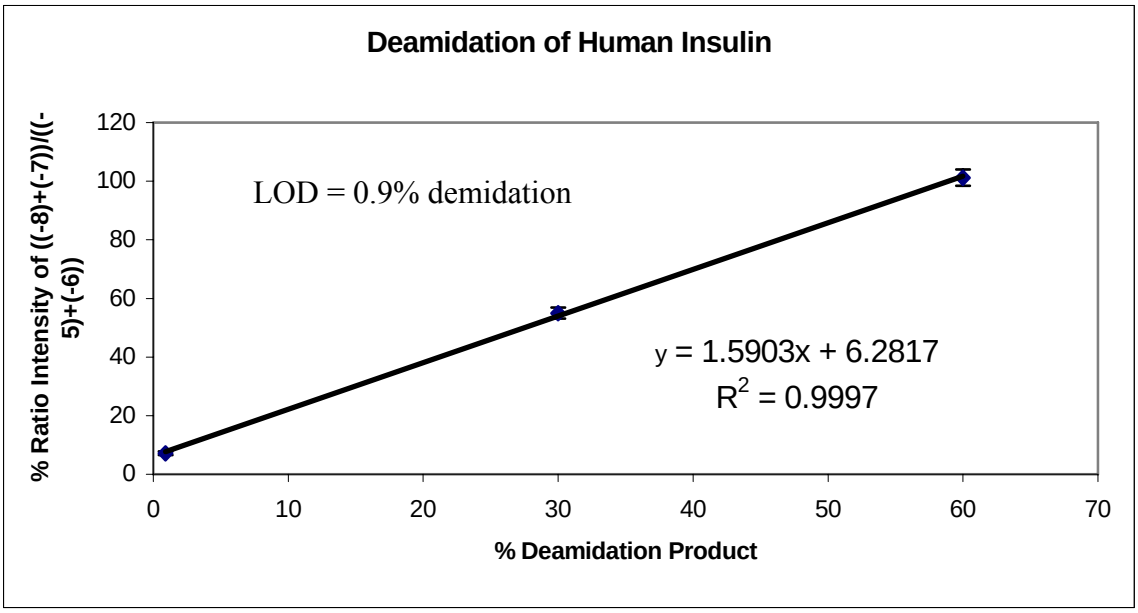

Figure 4.9 A plot between the intensities charge state (-8) + (-7) over (-5) +(-6) vs % deamidation product.

A plot of the % relative intensity of charges states**.** (–8) + (–7) over charge state (-5) + (–6) vs % deamidation product yielded the best straight line with the best R^2, small <CV> and RMSE and LOD = 0.9% deamidation product, Figure 4.9.

A plot of the ratio of intensities for charge state –7 to the sum of those for –5 and –6 vesus % the deamidation product (from HPLC) also shows good linearity and precision in Figure 4.10. Set 2 was chosen with a lower range of the deamidation product concentration and more data points. There are more possibilities to derive the calibration curves because small deamidation product samples were monitored in this set of the mass spectra. The calibration curves for this set are shown in Appendix 2 and summarized in Table 4.5.

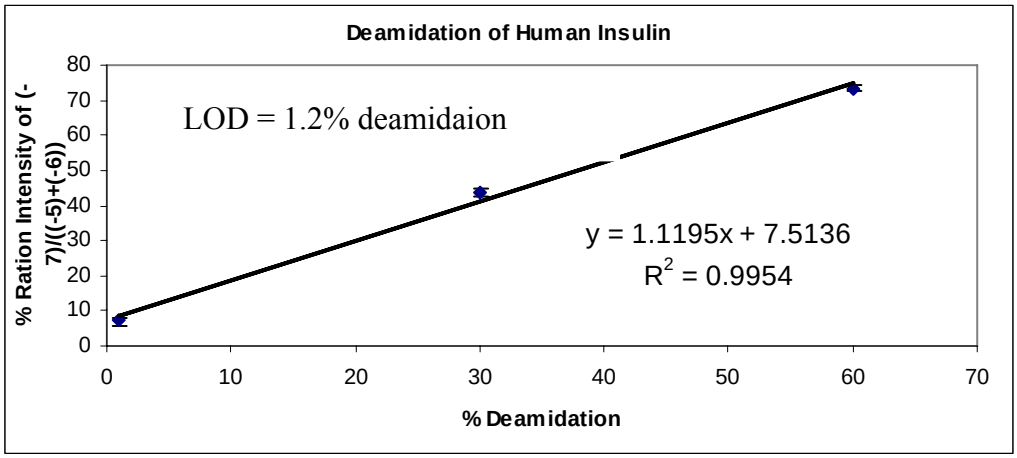

Figure 4.10 A plot between the intensities charge state (-7) over (-5) +(-6) vs % deamidation product

Table 4.5 Summary of quantitation performances of human insulin in Set 2

Numerator	Denominator	LOD	CV	RMSE	R^2	Plot Slope
7+8	5	0.7	3.56	38.0	0.9904	-
7+8	6	1.4	2.30	21.1	0.9699	2.30
7+8	5+6	0.6	2.17	10.2	0.9691	1.87
7+8	4+5+6	0.6	2.26	10.0	0.9651	1.86
7+8	5+6+7	0.7	1.70	6.6	0.9781	1.34
7+8	5+6+7+8	1.2	3.90	14.9	0.9793	1.27
7	5	0.8	4.0	37.1	0.9941	4.75
7	6	1.9	2.52	20.7	0.9816	1.69
7	5+6	0.8	2.57	10.7	0.9673	1.49
7	5+7	0.7	2.25	13.7	0.9687	2.29
7	5+6+7	0.9	2.02	6.9	0.9811	1.05
7	5+6+7+8	0.9	2.06	6.9	0.9834	0.99

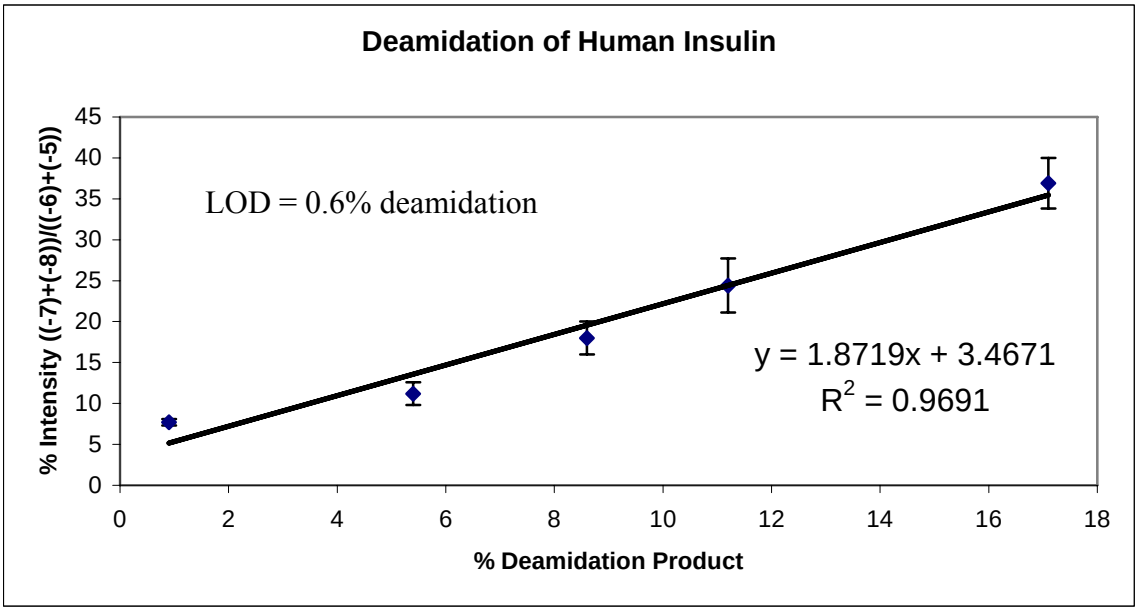

Figure 4.11 A plot between the intensities charge state (-8) + (-7) over (-5) +(-6) vs %

For example, a plot between the percent relative intensity of charges states (–8) + (–7) over charge state (-5) + (–6) and % deamidation product yield a straight line with LOD = 0.6% deamidation product but high value of the standard deviation, Figure 4.11.

In another example from set 2 (Figure 4.12), the calibration curve was % Intensity ((-7)+(-8)) / ((-7)+(-6)+(-5)) vs % deamidation product, yield the best fit with LOD = 0.7% deamidation. The stability of the charge states in set 1 is better than those in set 2. This is because the source capillary diameter (i.d.) in set 1 (0.05 mm) is smaller than in set 2 (0.12 mm). Therefore, the efficiency and the uniformity of the spray in set 1 were better. If the diameter of the capillaries were the same the result of set 2 would be better.

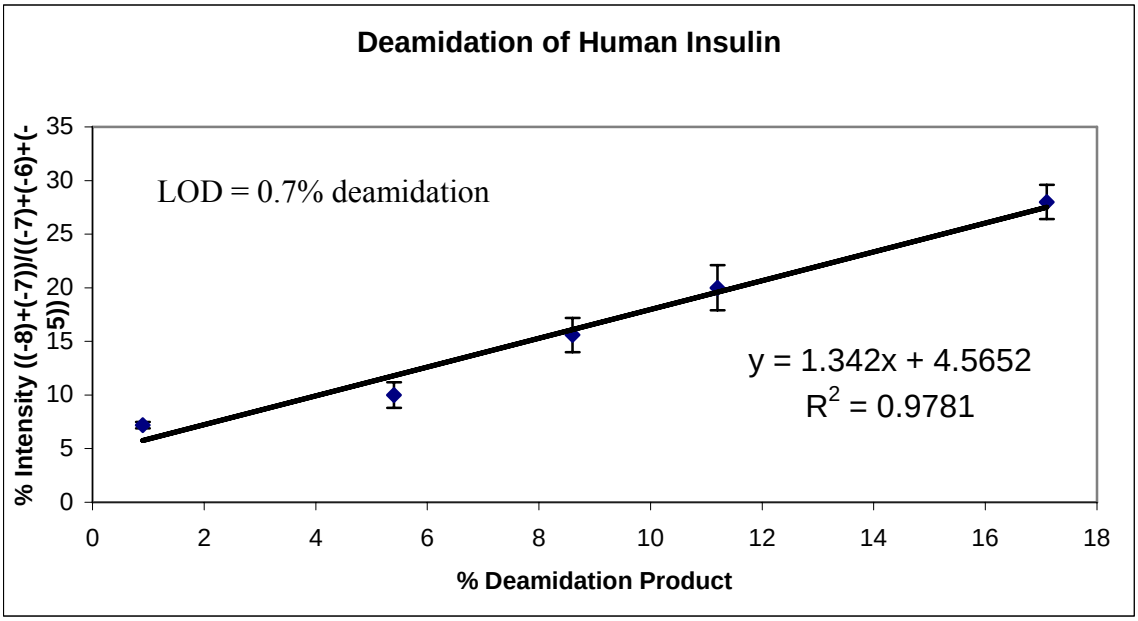

Figure 4.12 A plot between the intensities charge state (-8) + (-7) over (-5) +(-6) + (-7) vs % deamidation product set (2).

CHAPTER 5

CONCLUSIONS

5.1. Application of Gas Phase Isopropylamine in ESI to Determine Ratio of Human Insulin and Its Deamidation Product

Gas phase isopropylamine with negative ESI enable to differentiate the charge states belonging to human insulin and it deamidation product. This is because the deamidation product has more of carboxylic acid groups. The intensities of the charge states, expecially –7 and –8 charge states can be used to determine the ratio of human insulin and its deamidation product. Accuracy, precision, limit of detection (LOD), and linearity depend on which charge states are considered. The best fit, which plots of the relative intensity of ((-8) + (-7)) / ((-7) + (-6) + (-5)) vs % deamidation product yields a straight line, LOD = 0.7% deamidation.

5.2. Future Work

This method will be applied to real samples in pharmaceutical company to determine the ratio of human insulin and its deamidation product.

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APPENDIX

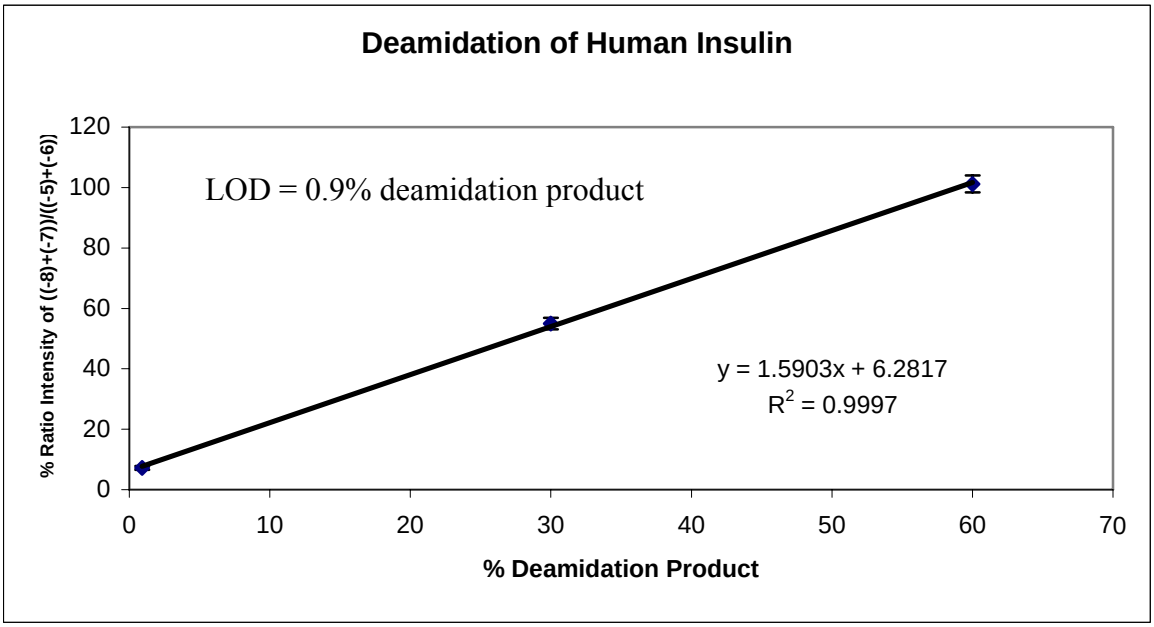
Appendix 1

Table A1 Experimental numerical data for Set 1

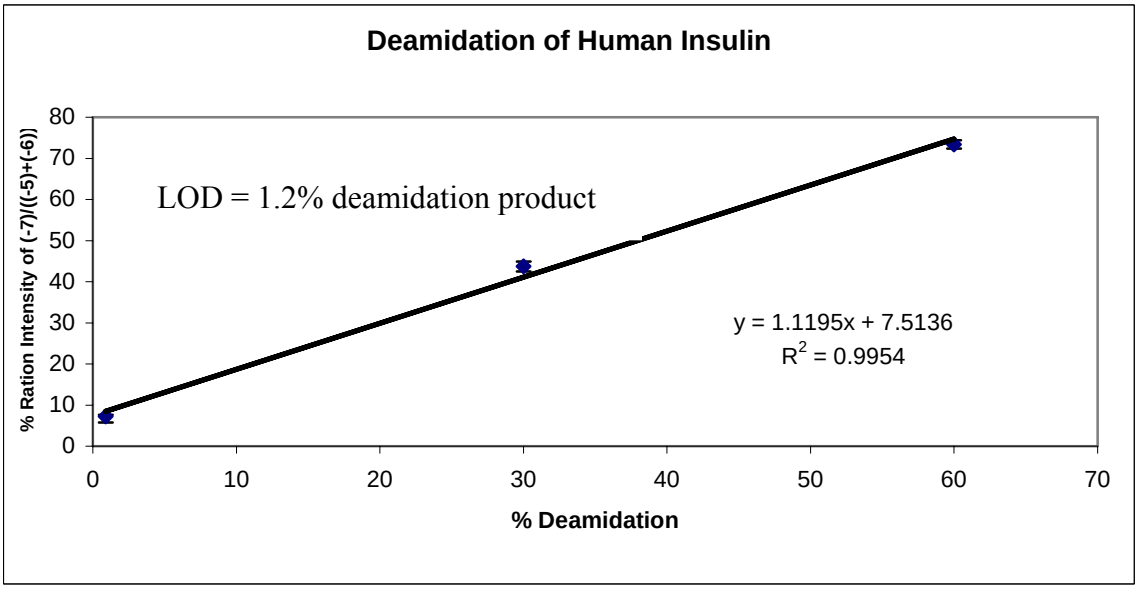
Ratio of the deamidation	charge -8	charge -7	charge -6	charge -5
1	0	930752	5805312	9098240
1	0	824064	4995072	7076608
1	0	988544	5990912	8007936
1	0	1080000	6347776	9115648
1	0	905600	5306880	7076608
1	0	1017792	5736192	7952384
1	0	1125760	6310144	8177152
1	0	1114688	6185472	8326912
1	0	1038016	5719296	8483840
1	0	1101632	6035200	8403968
30	4749568	17240064	26531840	14751744
30	2518272	10402816	15898624	9013248
30	2366976	10676224	16206848	8277760
30	2866944	11963392	17587200	10141696
30	3955456	15437824	22484992	12223488
30	3599104	13217792	19234816	10650624
30	4386048	16625664	24150016	14016512
30	4819200	17550336	25367552	14633984
30	4989184	18200576	26153984	14010368
30	3572736	13234176	18827264	10650624
60	22152192	63979520	65105920	23493632
60	23121920	64372736	63168512	23818240
60	23793664	64913408	65302528	24071168
60	24822784	65048576	65781760	24043520
60	24609792	66609152	66859008	24707072
60	26503168	67637248	65544192	25116672
60	26735616	67203072	66387968	24603648
60	26098688	68739072	66080768	25121792
60	25591808	66174976	65802240	25078784
60	26558464	66519040	64737280	25472000

Calibration Curves for % deamidation of human insulin of Set 1.

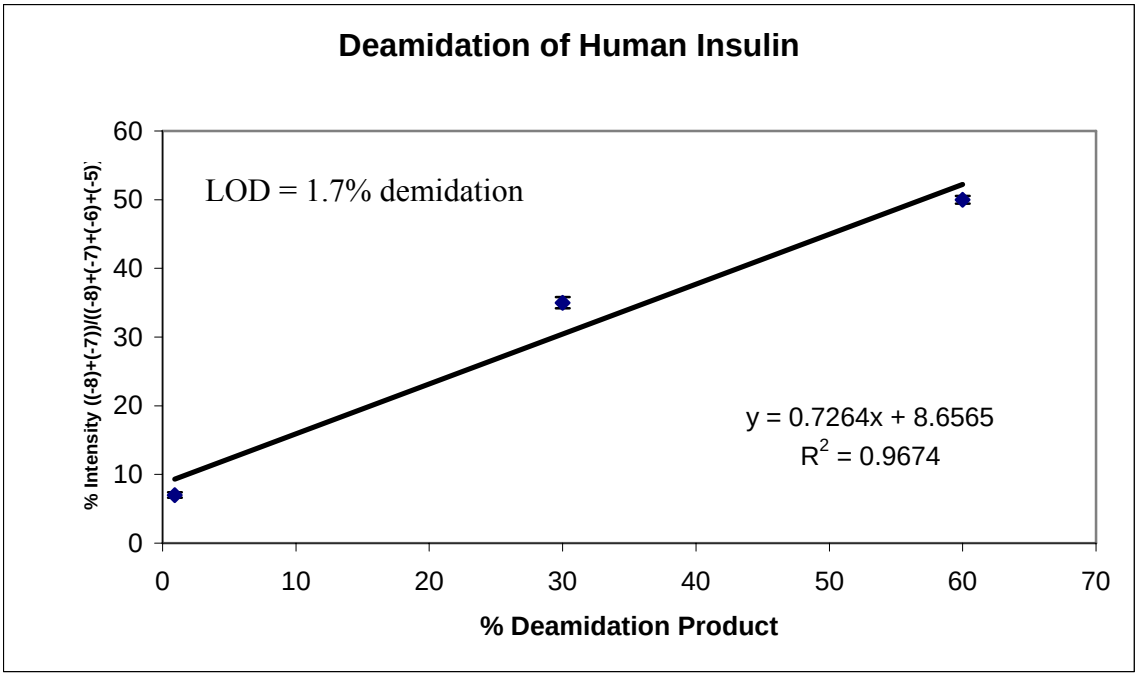
1. % Intensity ((-8)+(-7))/((-5)+(-6))



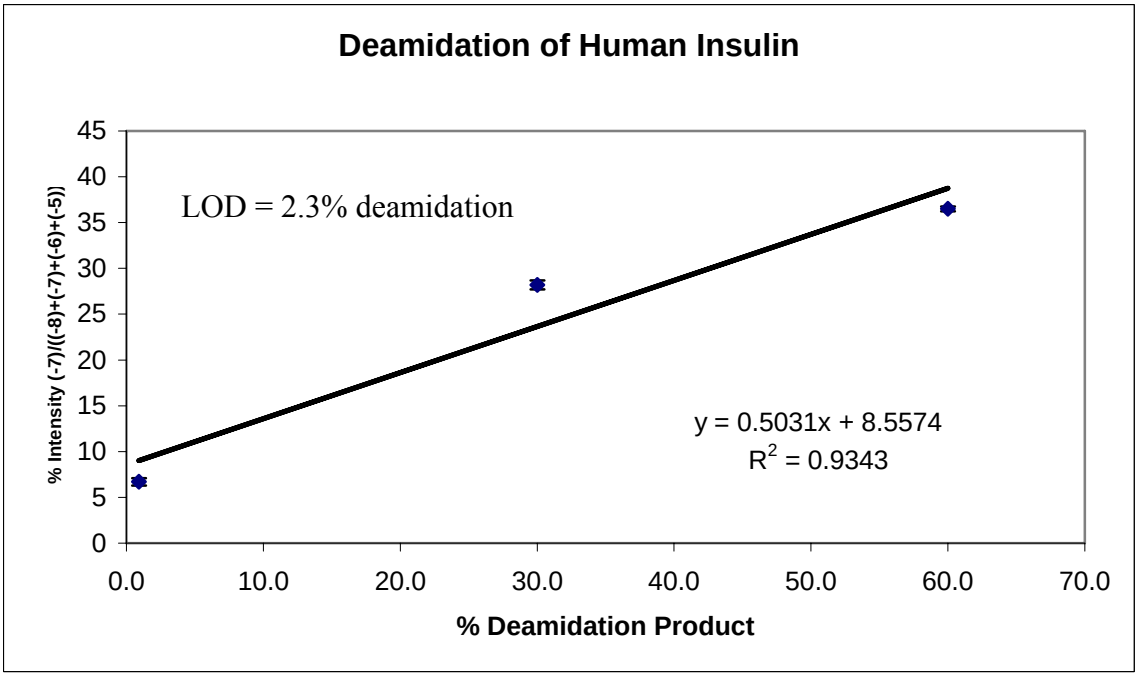
2. % Intensity (-7)/((-5)+(-6))



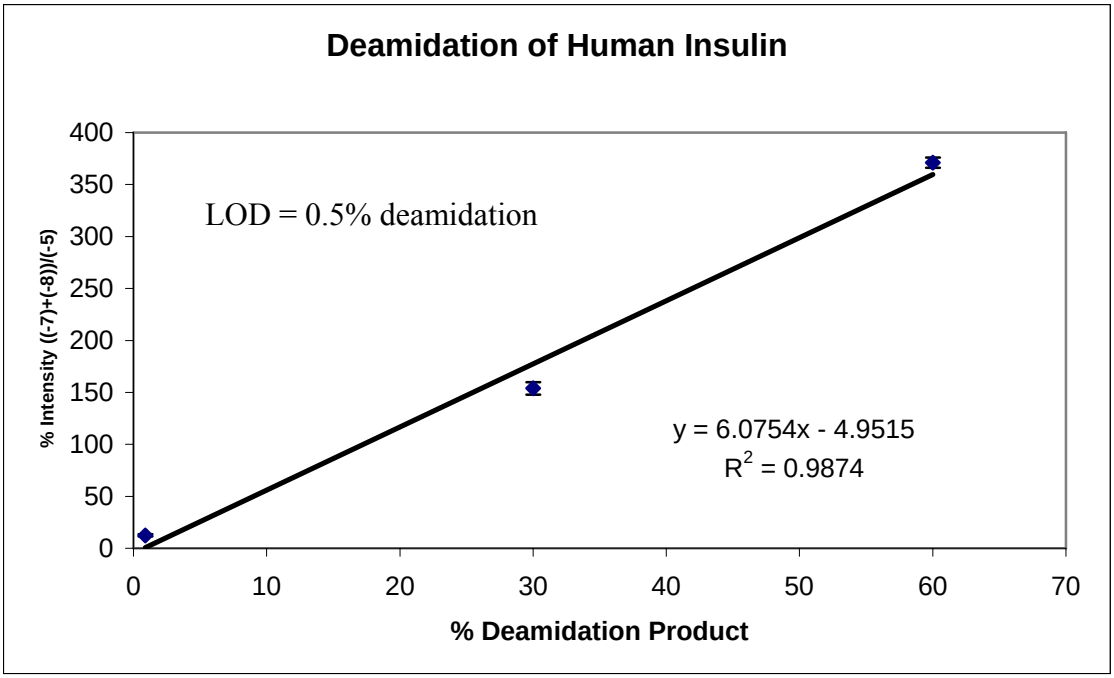
3. % Intensity ((-8)+(-7))/((-8)+(-7)+(-6)+(-5))



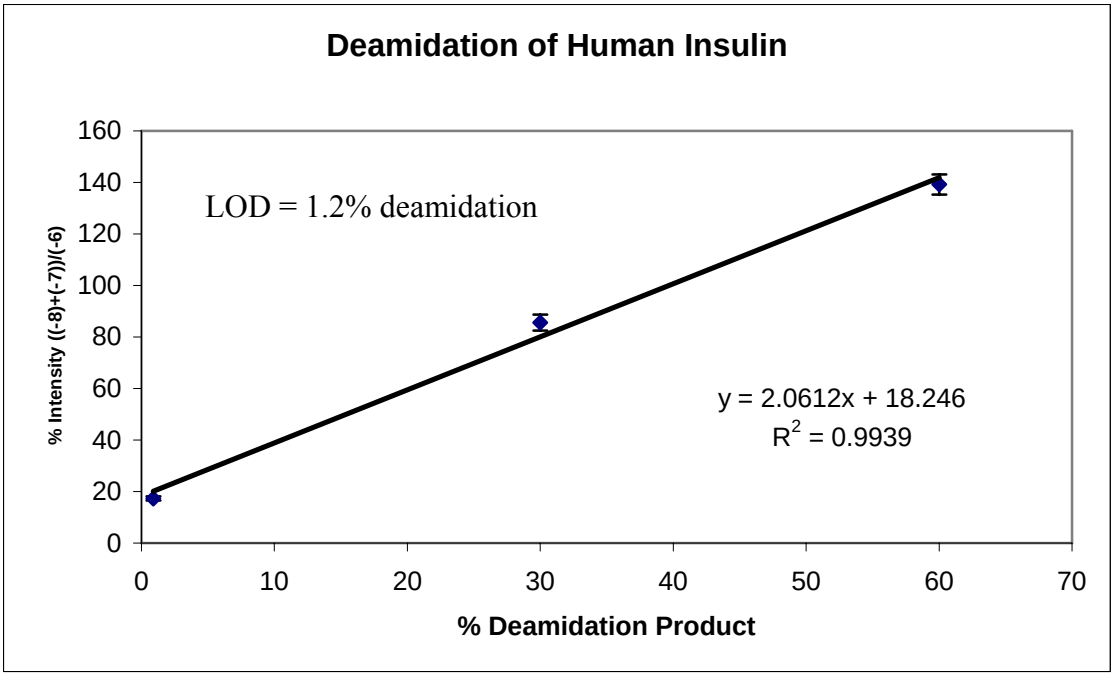
4. % Intensity (-7)/((-8)+(-7)+(-6)+(-5))



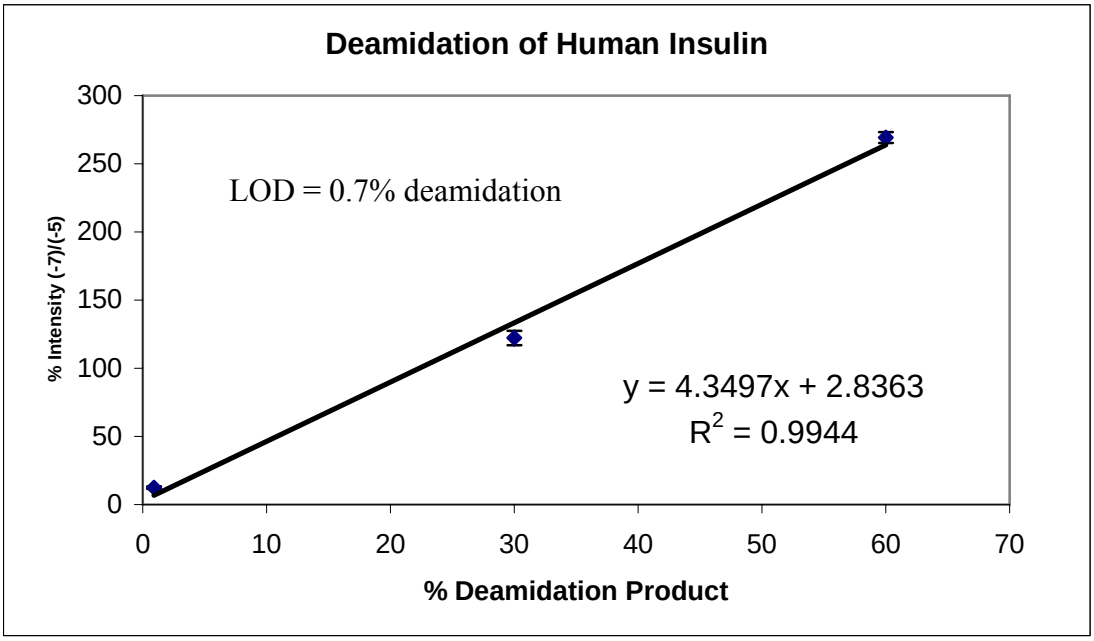
5. % Intensity ((-7)+(-8))/(-5)



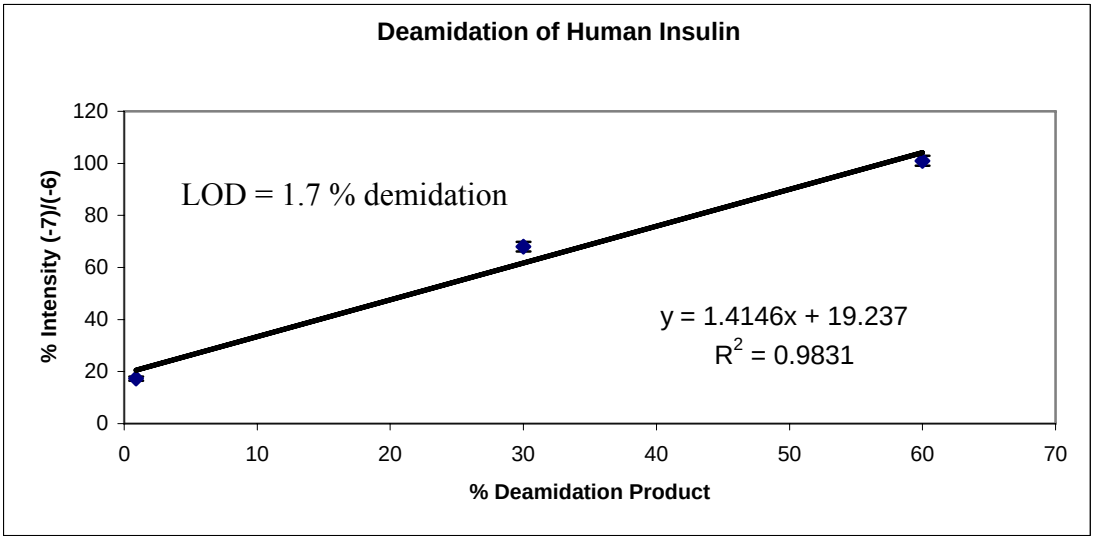
6. % Intensity of ((-7)+(-8))/(-6)



7. % Intensity of (-7)/(-5)



8. % Intensity of (-7)/(-6)



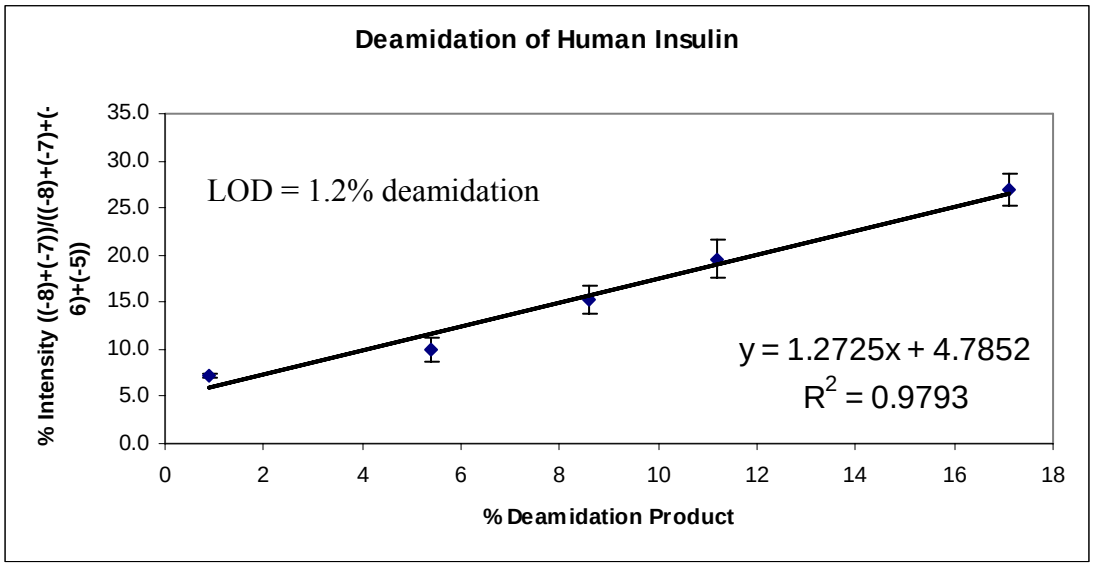
Appendix 2

Table A2 Experimental numerical data for Set 2

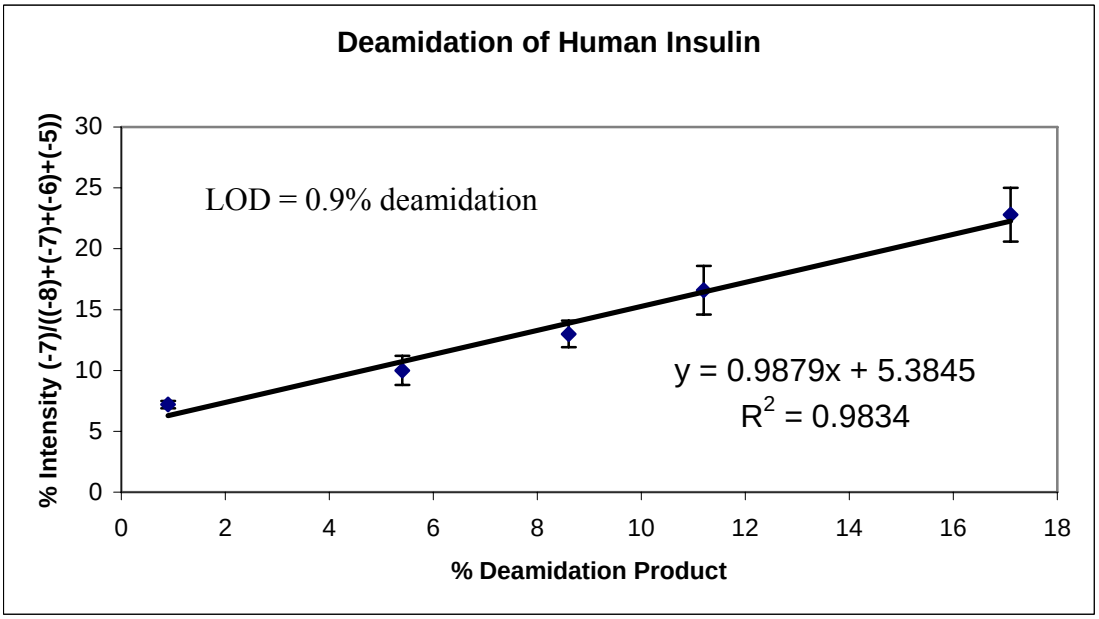
% deamidation		Relative	Intensity	of	Charge states
	-8	-7	-6	-5	-4
0.9		278656	1223040	2413312	791808
0.9		303968	1317824	2465280	829568
0.9		308944	1289475	2595328	833024
0.9		257744	1128384	2124032	783616
0.9		233024	1114240	2144512	793536
5.4		1308800	4540352	6054144	1096768
5.4		1177024	4873984	6344960	1360960
5.4		1001984	4542976	6505728	1670016
5.4		1193920	4394752	6098688	1468544
5.4		1343232	4281088	6403328	1510656
8.6	117752	790656	2836224	3291392	765760
8.6	219712	953152	2709504	3298048	707520
8.6	164272	1079872	3080960	3743744	1010944
8.6	200112	1147136	3636736	4123940	1051904
8.6	217520	1356736	3795712	4112640	1148160
11.2	248080	1548416	4418048	3303424	541248
11.2	394960	1855296	5588992	4662784	899904
11.2	206688	1353280	3048960	3637760	435920
11.2	142290	931648	2144768	1418816	210496
11.2	373168	1760960	4660736	4499968	502624
17.1	72116	316992	794304	405056	55732
17.1	79720	350192	747776	446160	49880
17.1	65536	353712	712960	426704	57776
17.1	71088	513296	1065344	460928	76504
17.1	57196	433872	715584	486976	40080

Calibration Curves for % deamidation of human insulin of Set 2.

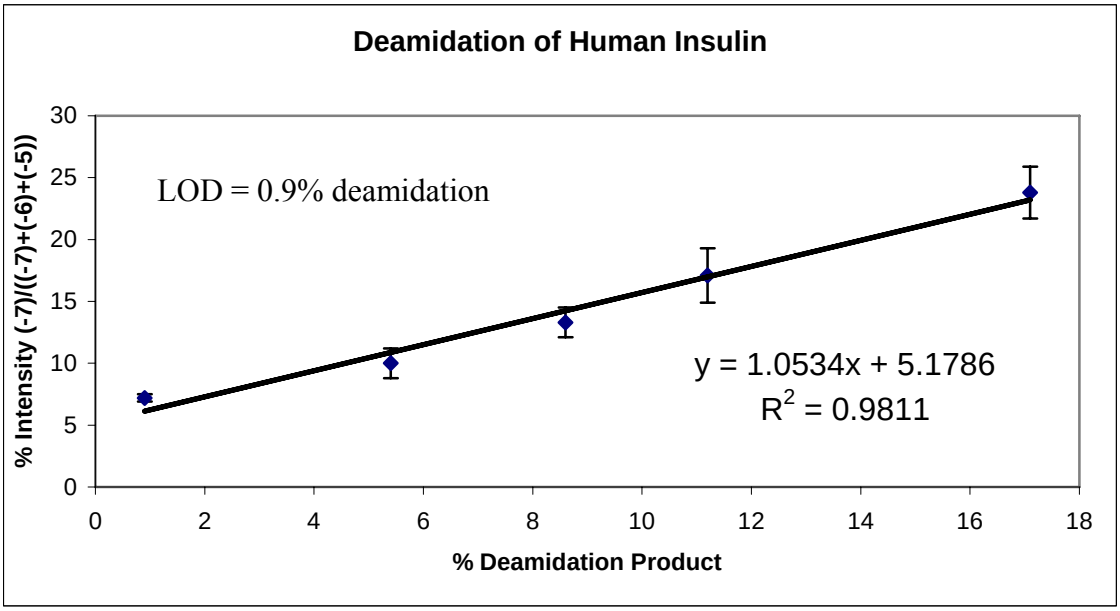
1. % Intensity ((-8)+(-7))/((-8)+(-7)+(-6)+(-5)



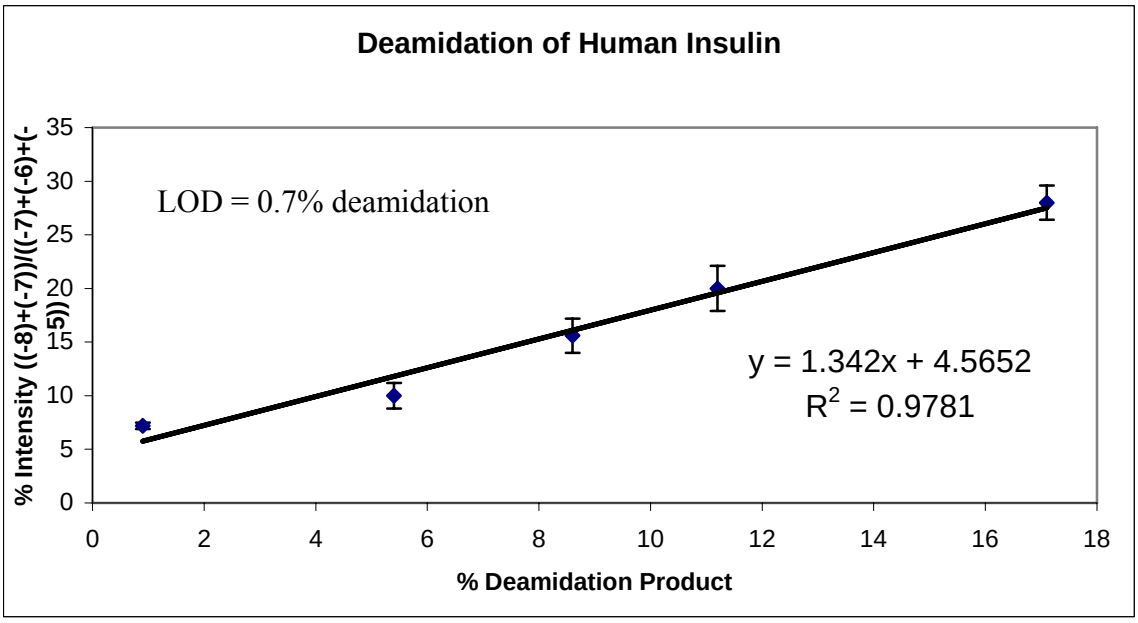
2. % Intensity (-7)/((-8)+(-7)+(-6)+(-5))



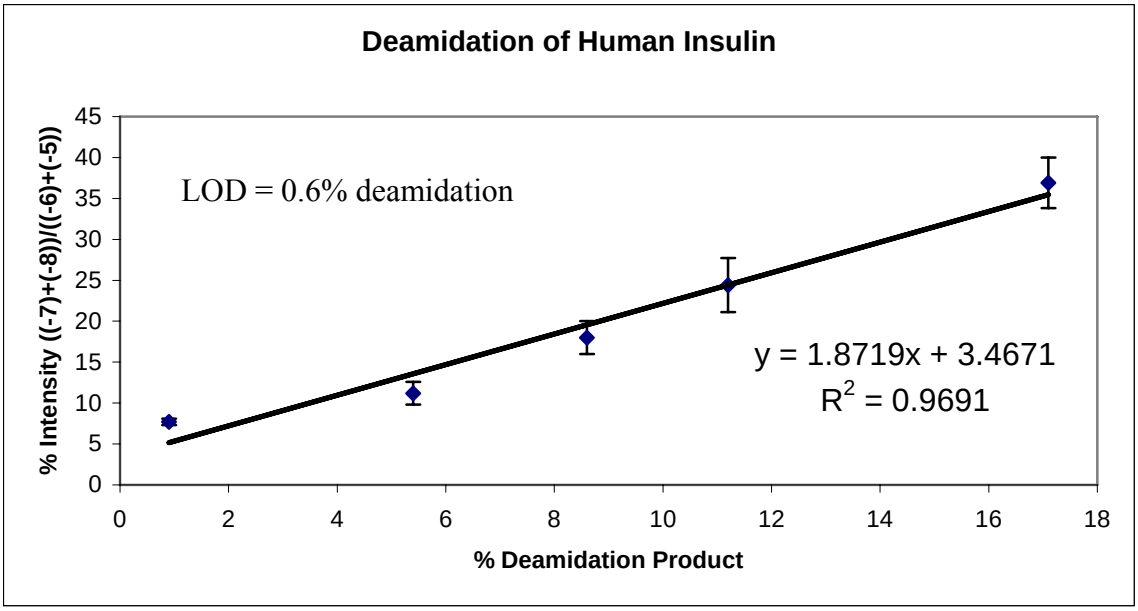
3. % Intensity (-7)/((-7)+(-6)+(-5))



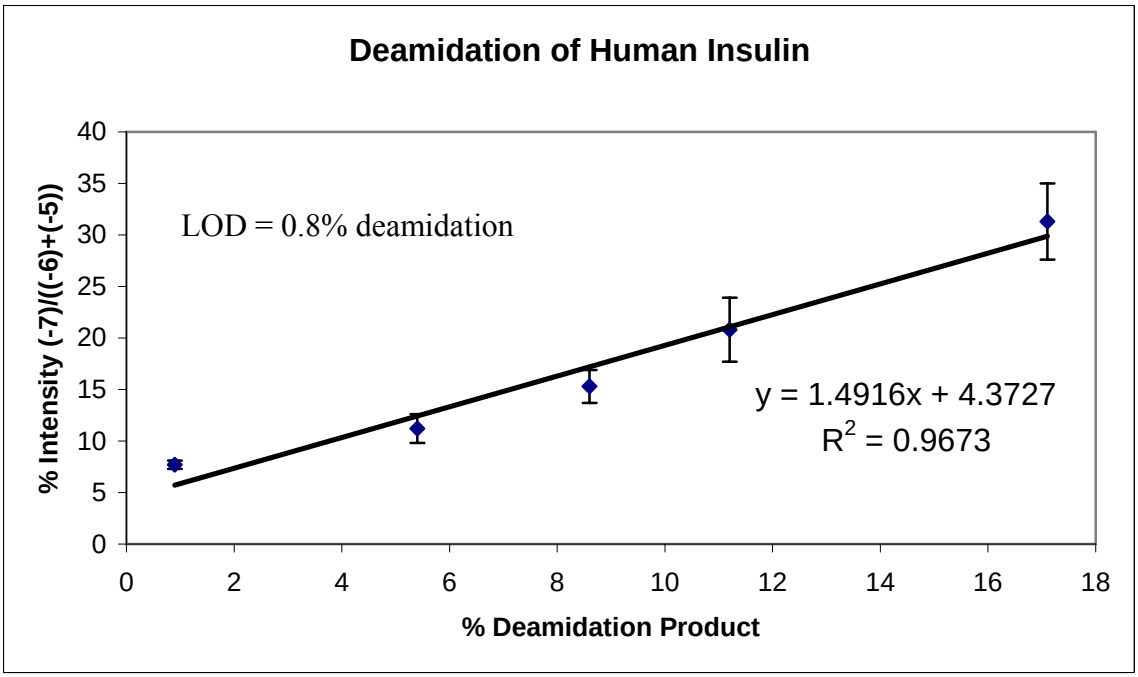
4. % Intensity ((-7)+(-8)) / ((-7)+(-6)+(-5))



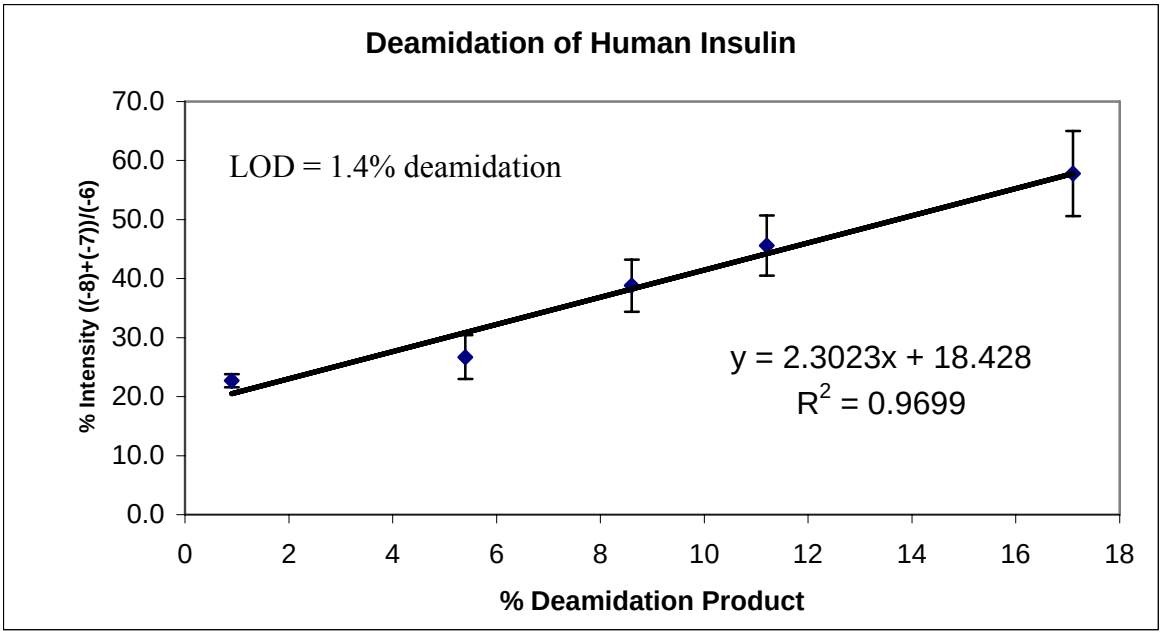
5. % Intensity of ((-7)+(-8))/((-6)+(-5))



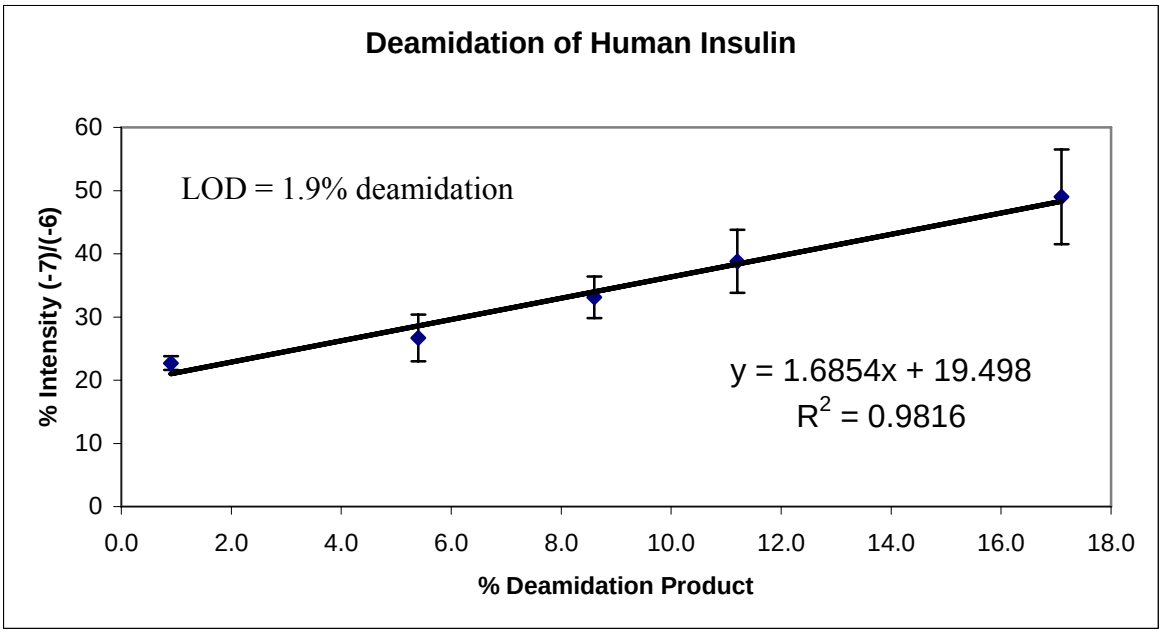
6. % Intensity (-7)/((-6)+(-5))



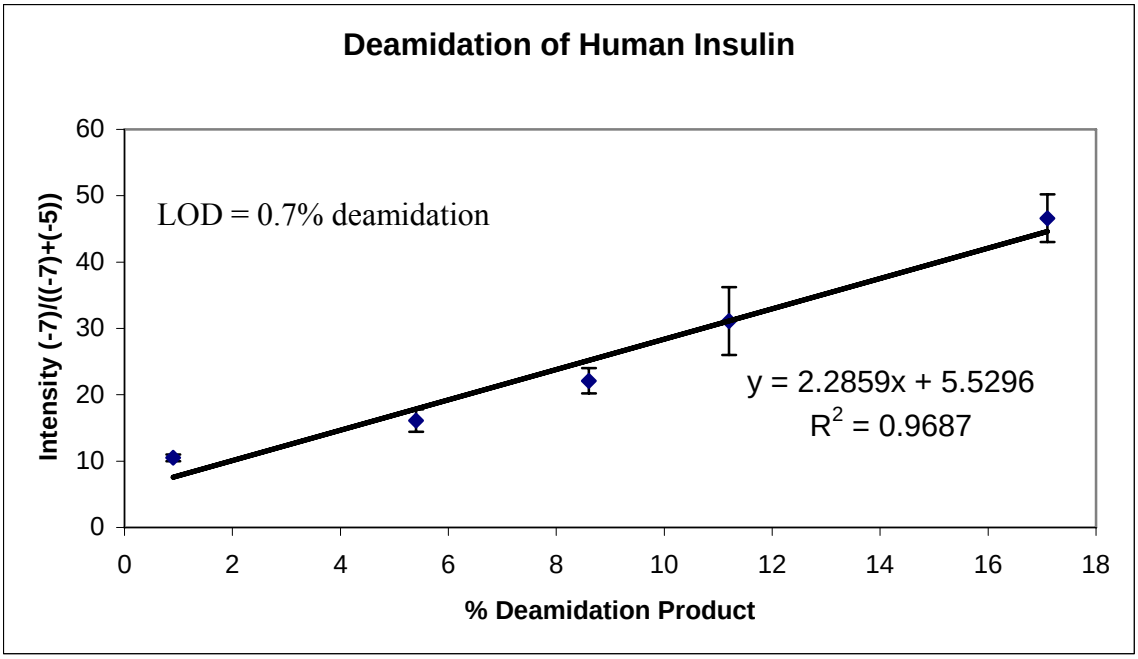
7. % Intensity ((-8)+(-7))/(-6)



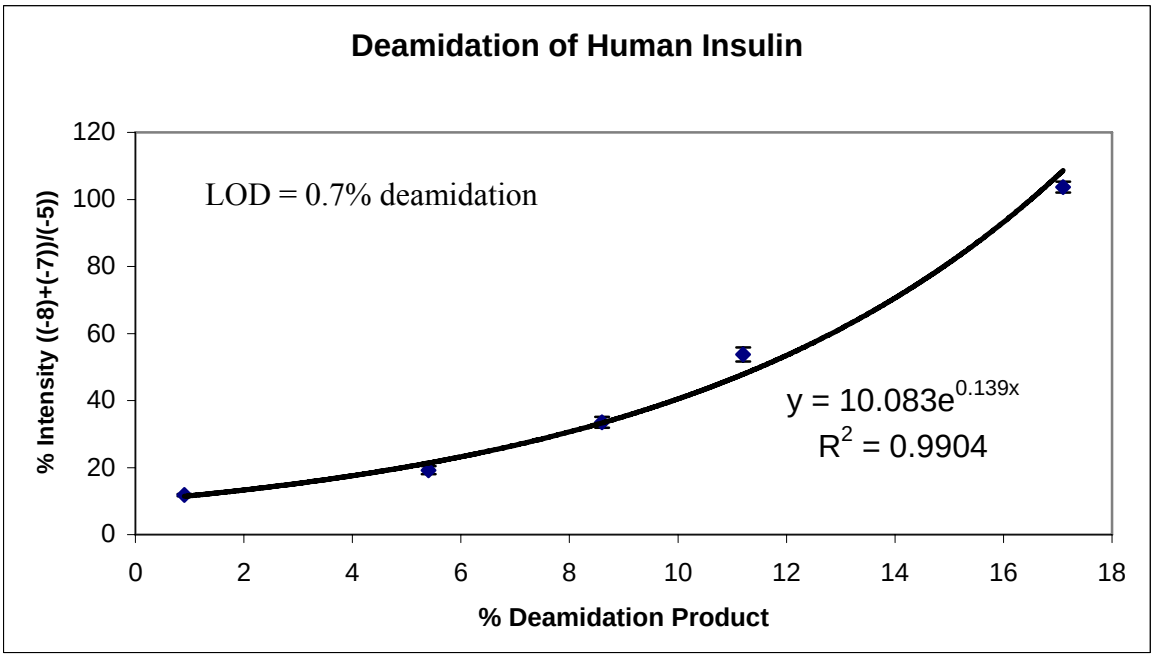
8. % Intensity (-7)/(-6)



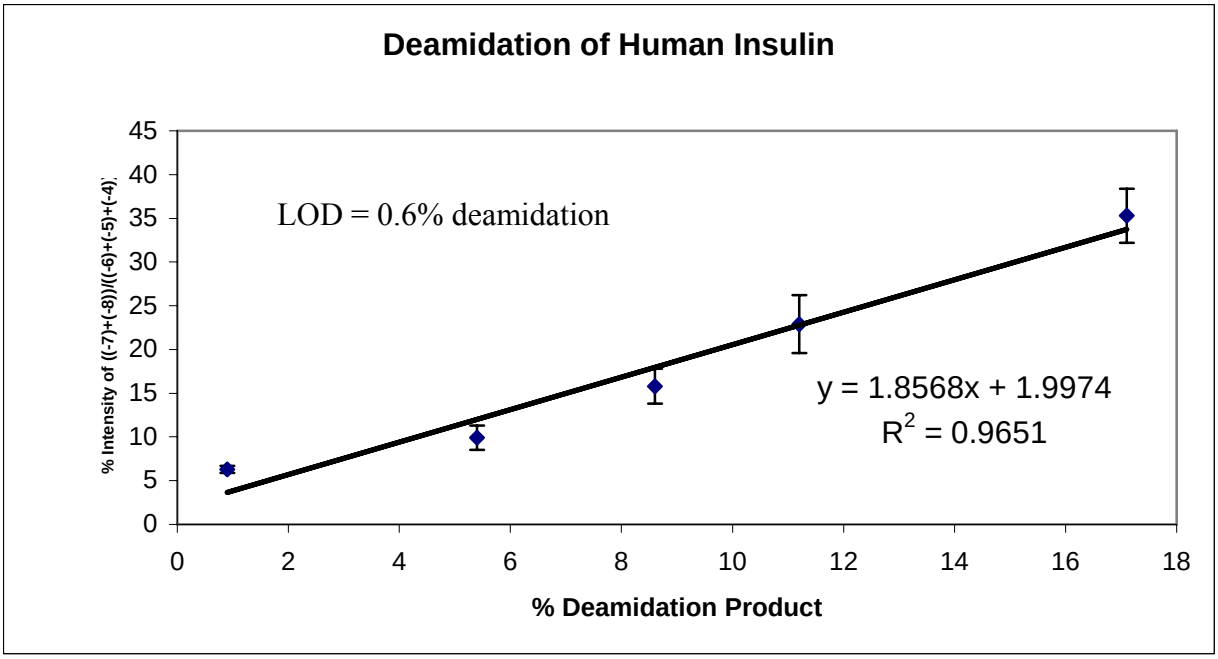
9. % Intensity (-7)/((-7)+(-5))



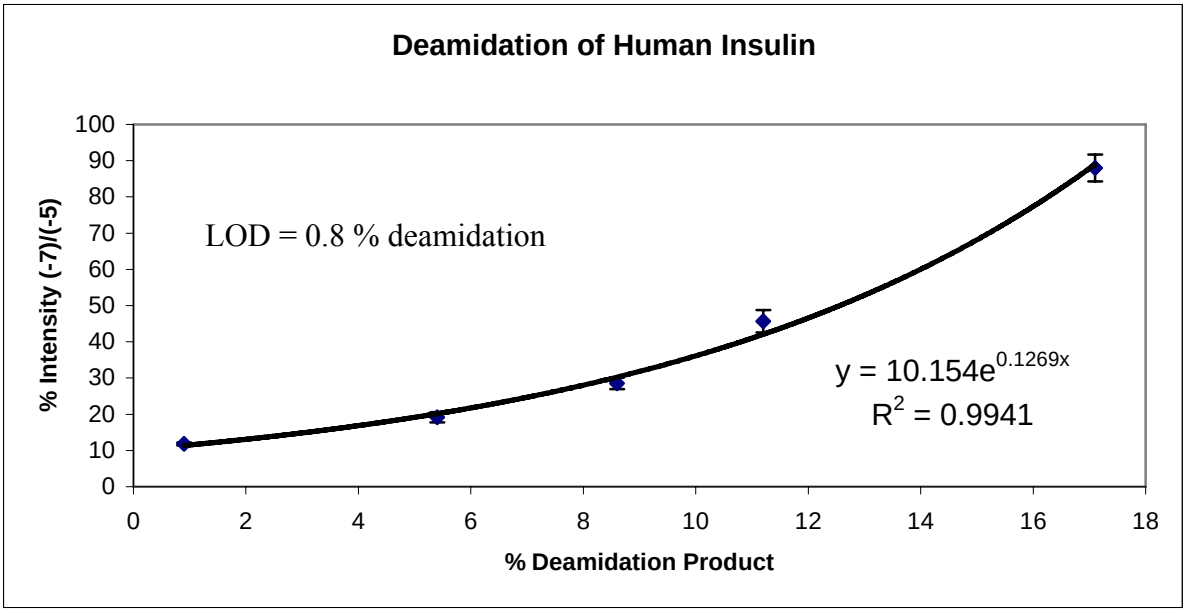
10. % Intensity ((-7)+(-8)) / (-5)



11. % Intensity of ((-7)+(-8))/((-6)+(-5)+(-4))



12. % Intensity (-7)/(-5)



VITA

Teerapat Rojsajjakul was born in Thailand, received his first master's degree in Organic Chemistry, focused on organic synthesis from Chulalongkorn University, Bangkok, Thailand and his second master's degree in Bioanalytical Chemistry, focused on hydrogen exchange – mass spectroscopy, from The University of Nebraska at Lincoln and his third master's degree in Analytical Chemistry, focused on mass spectrometry, at The University of Tennessee at Knoxville.